TRANSCRIPTIONAL CONTROL OF INTERNEURON DEVELOPMENT IN THE CENTRAL NERVOUS SYSTEM

by

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To Herb Harris, who always knew it would happen

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Table of Contents

List of Figures

Figure

Abstract

TRANSCRIPTIONAL CONTROL OF INTERNEURON DEVELOPMENT
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This work addresses the role of two transcription factors in generation of specific neuronal subtypes in the developing central nervous system (CNS): the bHLH transcription factor Bhlhb5 in spinal cord and the paired-like homeobox gene Pitx2 in spinal cord and hindbrain development.

Neural circuits that control motor activities depend on spatially and temporally ordered generation of distinct classes of spinal interneurons whose genesis is poorly understood. The Olig-related transcription factor Bhlhb5 plays two central roles in this process. Bhlhb5 repressor activity acts downstream of retinoid signaling and homeodomain proteins to promote formation of dI6, V1, and V2 interneuron progenitors and their differentiated progeny. Bhlhb5 is required to organize the spatially-restricted expression of Notch ligands Jagged1, Dll1, and Dll4 that affect the formation of the interneuron populations adjacent to Bhlhb5^{+} cells and influence the global pattern of neuronal differentiation. Through these actions, Bhlhb5 helps transform spatial

information established by morphogen signaling into local cell-cell interactions associated with Notch signaling that control the progression of neurogenesis and extend neuronal diversity within the developing spinal cord.

Pitx2 is involved in the regulation of left-right asymmetry and development of numerous organs, including pituitary, eyes, teeth, palate, heart, and limbs. Pitx2 is expressed in forebrain, midbrain, hindbrain, and spinal cord during development, with pleiotropic neurotransmitter phenotypes and functions. In spinal cord, Pitx2 is expressed in a subset of V0 interneurons that can be further subdivided by neurotransmitter phenotype. In hindbrain, Pitx2 is expressed in a discrete set of neurons in ventral rhombomere 1 that are GABAergic and may participate in local regulatory circuits.

In addition to contributing to the understanding of normal developmental processes, elucidation of roles of proteins like Bhlhb5 and Pitx2 can provide valuable direction for designing therapeutic treatments for devastating neurological diseases. Enthusiasm for stem cell research comes from the promise of pluripotent neural stem cells to generate the many types of neurons damaged or missing in disease. Thus, a better understanding of processes that recapitulate normal development can be vital for developing therapies that use neural stem cells for CNS regeneration and repair.

Chapter 1

Introduction

During central nervous system development, thousands of distinct types of neurons and glial cells in precise numbers and locations are produced from an initially homogeneous sheet of neuroepithelial cells. One of the fundamental questions in developmental neurobiology is how this precise specification is accomplished. Errors in this process can result in a wide range of disorders, from devastating developmental abnormalities resulting in perinatal lethality or severe lifelong disability to more subtle deficits in learning, attention, cognition, and affect. An understanding of the molecular mechanisms that control the process of differentiation of neural progenitor cells into the mature cell types and circuitry that form the vertebrate central nervous system (CNS) during development can provide insight into how this process occurs in normal and disordered development. Such insights are likely to suggest targets for prevention and treatment of the disorders that result when the process goes wrong.

A critical component of embryonic CNS development is the formation of segments or compartments that divide the neuroepithelium into discrete domains of gene expression that give rise to specific neuronal cell types. Positional information is established very early along the neural tube through secreted signaling factors that establish anterior-posterior and dorsal-ventral axes that then define discrete

neurodevelopmental regions (Briscoe and Ericson, 1999; Wilson and Edlund, 2001; Vieira et al., 2010). Along the anterior-posterior axis, the anterior portion of the neural tube becomes subdivided into three regions that will become the forebrain, midbrain and hindbrain; the posterior part of the neural tube becomes the spinal cord. Each of these gross divisions is further compartmentalized into discrete neurogenic regions, such as prosomeres in the telencephalon and rhombomeres in the rhombencephalon, each with defining profiles of transcription factors and giving rise to discrete categories of neurons.

The vertebrate spinal cord represents a structurally simple subdivision of the central nervous system that can be used to study the processes by which neural progenitor cells give rise to many distinct subclasses of motor neurons, interneurons, and glial cells in a spatially and temporally restricted pattern that in turn form the complex circuitry necessary for coordinated motor behavior (Goulding, 2009; Grillner and Jessell, 2009). In the developing spinal cord, functionally distinct classes of neurons are produced from specific progenitor domains established along the dorsal-ventral axis (Jessell, 2000; Poh et al., 2002). Multiple intersecting factors cooperate to determine these domains and regulate neurogenesis and cell fate specification within them. These include gradients of morphogens secreted from the floor plate, roof plate, and somitic mesoderm; homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors; and intercellular signaling such as the Notch signaling pathway.

Patterning of the spinal cord during embryonic development

The dorsal-ventral axis of the developing spinal cord is initially established by secreted factors from non-neural tissue. Bone morphogenetic proteins (BMPs) are

secreted from ectoderm that lies dorsally above the neural tube (Lee and Jessell, 1999) and Sonic hedgehog (Shh) is secreted from the notochord, a population of mesodermal cells that extends ventrally under the neural tube (Briscoe and Ericson, 1999). These secreted morphogens lead to the formation of the roof plate and floor plate, respectively, within the neural tube proper, which then become signaling centers themselves and establish the dorsal-ventral axis (Lee and Pfaff, 2001). A third morphogen, retinoic acid (RA), is secreted from the paraxial mesoderm that lies alongside the neural tube, and contributes to the formation of interneuron progenitor domains within the intermediate spinal cord (Pierani et al., 1999) as well as to the formation of motor neurons (Novitch et al., 2003).

In the ventral spinal cord, secretion of Shh by the floor plate results in a concentration gradient that extends dorsally (Fig. 1.1A). This gradient supplies positional information along the dorsal-ventral axis and regulates expression of specific homeodomain (HD) transcription factors in a precise spatial and temporal manner. These HD proteins can be divided into two classes on the basis of their regulation by Shh: class I proteins are induced by RA signaling and are repressed by distinct concentrations of Shh whereas class II proteins are induced in a concentration-dependent manner and require Shh for their expression (Pierani et al., 1999; Briscoe and Ericson, 2001; Novitch et al., 2003). Repressive interactions between pairs of Class I and Class II proteins serve to translate graded Shh signaling into sharp boundaries that define discrete progenitor domains. Class I proteins include the more dorsally expressed Pax7, Irx3, Dbx1, Dbx2, and Pax6. Class II proteins include the more ventral Nkx6.1, Nkx6.2 and Nkx2.2 (Briscoe et al., 2000).

The combinatorial expression of these proteins and cross-repressive interactions between them define five distinct progenitor domains in the ventral neural tube (from ventral to dorsal: p3, pMN, p2, p1, and p0), each of which give rise to distinct classes of neurons (Fig. 1.1B). For example, the HD proteins Nkx6.1 (class II) and Dbx2 (class I) are expressed in non-overlapping domains and the common border of their expression domains defines the p1/p2 domain boundary. Ectopic expression of *Dbx2* ventrally or of *Nkx6.1* dorsally each represses expression of the other in a selective and cell-autonomous manner (Briscoe et al., 2000). Furthermore, the subtype identity of differentiated neurons emerging from these domains changes in accordance with the modified complement of HD factors created by gain-of-function and loss-of-function manipulations. This indicates that neuronal subtype identity is initially established by the combinatorial action of transcription factors present in discrete progenitor domains, i.e., a HD code that defines each domain (Briscoe et al., 2000; Jessell, 2000; Briscoe and Ericson, 2001; Pierani et al., 2001). The dependence of HD patterning proteins on particular concentrations of Shh for expression is confirmed by studies showing that progressively ventral cell fates can be induced in the presence of increasing concentrations of Shh (Litingtung and Chiang, 2000; Briscoe and Ericson, 2001).

Although Shh signaling is necessary and sufficient to induce ventral progenitor domains, its activity is both direct and indirect. Loss of Shh prevents formation of the floor plate, p3, pMN, and p2 domains and results in displacement of the p1 and p0 domains to the most ventral part of the neural tube (Chiang et al., 1996; Briscoe and Ericson, 1999; Jessell, 2000; Ruiz i Altaba et al., 2003). When all Shh signaling is blocked by removal of smoothened (Smo), which is required for cells to respond to

hedgehog signaling (Fig. 1.2), none of the ventral progenitor domains form with the exception of some p0 character cells in the most ventral positions (Briscoe et al., 2001; Wijgerde et al., 2002). Conversely expression of a constitutively active Smo expands the creation of ventral cell types (Hynes et al., 2000). Patched1 (Ptc1) acts as a repressor of Shh signaling (Fig. 1.2) and, as a target of Shh signaling itself, is expressed in a ventral to dorsal gradient (Briscoe et al., 2001). Overexpression of a constitutively active form of Ptc1 can inhibit ventral fates and induce ectopic intermediate fates, which are normally confined to the p2-p0 domains, in more ventral locations by inhibiting the level of Shh signaling to which ventral cells can respond (Briscoe et al., 2001).

Recent evidence has suggested that Shh-mediated induction of ventral neural fates is influenced by complex regulatory and feedback mechanisms involved in Shh signaling and the action of its effector proteins, particularly Gli proteins (Jacob and Briscoe, 2003). There are three Gli proteins in vertebrates, Gli1, Gli2, and Gli3, each with distinct expression patterns (Matise et al., 1998). *Gli1* expression is confined to the ventralmost region adjacent to the floor plate. *Gli2* is expressed in the ventral and intermediate regions whereas *Gli3* expression is normally restricted to the dorsal part of the spinal cord. Gli2 is a transcriptional activator in the presence of hedgehog signaling. In the absence of Gli2, the floor plate and V3 neurons fail to form and motor neurons are ectopically generated in the most ventral positions (Matise et al., 1998; Stamataki et al., 2005). Conversely, increasing levels of Gli activity have been linked to more ventral cell fates. Gli3 normally acts as a transcriptional repressor; however, the presence of hedgehog signaling prevents the proteolytic processing of Gli3 to its repressor form. In the developing neural tube, Gli3 expression is limited to the intermediate and dorsal

regions, areas that receive lower amounts levels of Shh ligand and therefore exhibit a higher level of Gli3 repressor activity than that seen in the ventral spinal cord (Litingtung and Chiang, 2000; Jacob and Briscoe, 2003). In *Gli3* mutants, which lack Gli3 repressor activity, ventral fates expand. Progenitor domains in the intermediate neural tube expand dorsally, suggesting that Gli3 repressor activity modulates Shh inductive signals in more dorsal domains (Persson et al., 2002). Interestingly, in *Shh* or *Smo* mutants that also carry a mutation in *Gli3* only the floor plate and the most ventral p3 domain fail to be specified. Neurons derived from p2, p1, and p0 domains are generated, although they are not confined to distinct progenitor populations but are intermingled in the ventral neural tube (Litingtung and Chiang, 2000; Wijgerde et al., 2002; Motoyama et al., 2003; Bai et al., 2004). This rescue of aspects of progenitor domains in the absence of both Shh and its repressor Gli3 suggests the existence of Shh-dependent and Shh-independent aspects of dorsal-ventral patterning of progenitor domains in the spinal cord.

The activity of Shh in concert with Gli proteins induces specification of different cell fates by integrating both positional and temporal information. This is accomplished through responses to both higher concentrations and longer exposures to Shh, both characteristic of cells located more ventrally near the floor plate and notochord (Stamataki et al., 2005). Indeed, duration of exposure as well as concentration of Shh appear to be important for induction of particular neuronal fates. For example, longer exposure to Shh signaling is needed for induction of pMN cell types than for p2-derived neurons (Poh et al., 2002; Dessaud et al., 2007; Dessaud et al., 2010).

In the intermediate ventral neural tube, both Shh and RA are involved in specification of progenitor domains p1 and p0. While low concentrations of Shh can

induce expression of the p1/p0 markers *Dbx1* and *Dbx2*, Shh signaling is not absolutely required for the generation of these cell types (Pierani et al., 1999). Treatment with RA efficiently generates *Dbx1/2* progenitors and their progeny and in its absence specification of p1 and p0 progenitor domains is impaired (Pierani et al., 1999). However, p0 neurons are still created in the complete absence of hedgehog signaling. These results indicate that the most dorsal of the ventral progenitor domains is dependent completely on RA rather than Shh signaling (Wijgerde et al., 2002), whereas pMN, p2, and p1 progenitor domains require both RA and Shh. RA signaling can induce the Class I HD proteins Pax7, Pax6, Dbx1, Dbx2, and Irx3 in the neural tube, working in concert with Shh induction of Class II and repression of Class I HD factors. Thus RA and Shh have both complementary and coordinate roles in progenitor domain specification (Diez del Corral et al., 2003; Novitch et al., 2003).

The bHLH transcription factor Olig2 in motor neuron generation

In addition to regulatory interactions between morphogen signaling systems and HD transcription factors in patterning of the ventral neural tube, cross-regulation also occurs between HD and bHLH transcription factors and contributes to differentiation of discrete neuronal cell types (Briscoe et al., 2000; Gowan et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Parras et al., 2002; Briscoe and Novitch, 2008). One of the first demonstrations of the role of bHLH transcription factors in the control of particular neuronal subtype identities in spinal cord was the discovery of the role of *Olig2* in motor neuron identity and differentiation (Mizuguchi et al., 2001; Novitch et al., 2001). Although cross-repressive interactions between Shh-induced class II and Shh-repressed

class I HD proteins define boundaries between adjacent progenitor domains and each progenitor domain is characterized by unique combinations of these factors (Briscoe and Ericson, 2001), not all boundaries can be defined by a class I-class II pair of HD transcription factors (Fig. 1.1). In particular, the pMN domain expresses the HD markers *Pax6*, *Nkx6.1*, and *Nkx6.2*. The ventral pMN/p3 domain boundary can be distinguished by the extent of expression of *Pax6* (class I) in the pMN domain and *Nkx2.2* (class II) ventrally in p3 progenitors. Dorsal to the pMN domain, the class I HD protein Irx3 is expressed by p2 progenitors, but a complementary class II HD protein is not evident (Briscoe et al., 2000). Therefore, the dorsal p2/pMN boundary can not be defined on the basis of HD protein expression. Interestingly, *Olig2*, a bHLH transcription factor, functions as the class II transcriptional repressor to establish the p2/pMN domain boundary in opposition to *Irx3*. It is also required for motor neuron formation and to direct acquisition of pan-neuronal character in prospective motor neurons (Mizuguchi et al., 2001; Novitch et al., 2001).

Originally recognized for its role in oligodendrocyte differentiation (Lu et al., 2000; Zhou et al., 2000), *Olig2* is expressed specifically in motor neuron progenitors shortly after neural tube closure and depends on the HD Nkx6 proteins for expression (Novitch et al., 2001). Misexpression of *Olig2* represses the HD protein Irx3 to low levels in the intermediate neural tube but less efficiently represses more dorsal expression. Conversely, misexpression of *Irx3* represses *Olig2* expression, suggesting that *Olig2* participates in the cross-regulatory interaction needed to establish the p2/pMN domain boundary in a manner analogous to Class I-Class II HD protein interactions. Misexpression of the p3 HD determinant Nkx2.2 represses Olig2 expression within the

pMN domain, however misexpression of *Olig2* does not lead to reciprocal repression of *Nkx2.2* (Novitch et al., 2001). Previous studies also established that formation of the pMN/p3 boundary is controlled by *Pax6* and *Nkx2.2* (Briscoe et al., 2000). Therefore, multiple and possibly redundant levels of control of progenitor domain specification operate within the ventral spinal cord during early development.

Complex interactions are also observed in *Olig2*-mediated cell fate specification in the ventral spinal cord. Misexpression of *Olig2* generates ectopic motor neurons (coexpressing *Isl1/2* and *Lhx3*) in areas of low *Irx3* expression, whereas V2 interneurons (coexpressing *Chx10* and *Lhx3*) are generated in areas of high *Irx3* expression, with a corresponding reduction in V0 and V1 interneuron markers. Some cells express markers of a hybrid motor neuron-V2 interneuron state, coexpressing *Isl1/2* and *Chx10* along with Lhx3, suggesting a contextual response to *Olig2* in downstream targets. *Olig2* also promotes expression of the pan-neuronal bHLH factors *Neurog2* and *NeuroD4* indicating a dual role in directing both a motor neuron specific fate and neurogenesis. Coexpression of *Olig2* and *Neurog2* induces ectopic motor neurons to a wider extent than misexpression of *Olig2* alone, demonstrating a powerful ability to override contextually determined neurogenic programs when both are expressed at high levels (Mizuguchi et al., 2001; Novitch et al., 2001).

Results from loss of function studies reveal that *Olig2* is required for motor neuron generation (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). In the absence of *Olig2*, no motor neurons form. Instead, expression of markers of V2 neurons, normally confined to a domain dorsal to *Olig2*, expand ventrally into the presumptive motor neuron domain. The HD protein Irx3 expands ventrally, consistent

with the role of *Olig2* in establishing the dorsal pMN boundary (Novitch et al., 2001; Zhou and Anderson, 2002). *Neurog2* is also not induced in the absence of *Olig2*, causing progenitor cells to migrate out of the ventricular zone without properly executing a differentiation program.

Results from these studies suggest that bHLH proteins like Olig2 contribute to cell fate specification in the developing neural tube in multiple ways. First, *Olig2* expression is controlled by signals that pattern other progenitor populations. Second, *Olig2* participates in the interactions that create and maintain particular progenitor populations. Third, *Olig2* directs formation of specific types of neurons. bHLH proteins are well known for their role in promoting general neuronal fates, but these recent observations showing that bHLH proteins have domain-restricted expression patterns imply roles in specification of particular cell types as well.

bHLH transcription factors in neural development

The bHLH family of transcription factors figures prominently in many developmental processes and in particular in neural development. bHLH genes were first recognized for their roles in vertebrate myogenesis and *Drosophila* neurogenesis (Jan and Jan, 1993; Massari and Murre, 2000). bHLH proteins share a common structure consisting of a basic region, required for DNA binding, and two amphipathic helices which are separated by a loop of varying length that participate in dimerization as well as in DNA binding (Fig. 1.3). The defining structure, the basic helix-loop-helix domain, has been used to identify many other members of this large and important family. bHLH proteins characteristically bind DNA at a consensus sequence known as an E-box

(CANNTG) (Murre et al., 1989) or more rarely to a modified E-box motif (CACNAG) known as an N box (Bramblett et al., 2002). Tissue and functional specificity are conveyed by regulation of hetero- or homo-dimerization and by availability of binding partners and posttranslational modifications rather than by DNA-binding sequence specificity (Bertrand et al., 2002; Conway et al., 2010). bHLH factors are hypothesized to play a central role in integrating positional and temporal information in the progression of neurogenesis precisely due to their ability to form functional interactions with a variety of locally expressed determinants.

Drosophila achaete-scute and *atonal* are prototypical exemplars of bHLH proneural proteins (Bertrand et al., 2002). Members of the achaete-scute complex (*asc*, including *achaete*, *scute*, *lethal of scute*, and *asense*) were identified as regulators of early steps of neural development and are required for generation of external sense organs (mechanosensory and chemosensory) and neuroblasts (Villares and Cabrera, 1987). Ectopic expression of *asc* genes induces the creation of neurons at the expense of epidermal cells, showing that they share proneural activity. Another bHLH gene, *atonal* (*ato*), was identified as being required for development of internal chordotonal organs (proprioceptors) (Jarman et al., 1993; Bertrand et al., 2002). In both cases, these bHLH genes are involved in promoting selection of neural progenitors from ectodermal cells and endowing them with general neuronal properties, defining them as proneural genes. However, they differ in their capacity to promote particular neuronal cell fates when overexpressed (Bertrand et al., 2002). These proteins have discrete patterns of tissue distribution, with achaete and scute involved in external sensory organ formation and atonal involved in chordotonal organ development. Both function as regulators of

neurogenesis through formation of heterodimers with the ubiquitously expressed bHLH protein Daughterless (Cabrera and Alonso, 1991; Lee 1997; Murre et al., 1989).

Orthologues of *Drosophila* proneural bHLH genes and additional members of the family important for vertebrate neurogenesis have been discovered based on sequence similarity within the characteristic basic domain using low stringency hybridization or PCR amplification with degenerate oligonucleotides in various tissue sources (Lee, 1997). These searches revealed a large family of over one hundred bHLH transcription factors, many of which have important developmental roles, especially in neurogenesis (Fig. 1.3).

Multiple functional and structural dimensions have been used to categorize the many bHLH proteins, including breadth of expression, neurogenic role, and mode of function. Based on specificity of expression, bHLH proteins can be separated into two major groups, class A proteins, which are ubiquitously expressed, and class B proteins, which are expressed selectively in specific tissues. Examples of class A proteins are *Drosophila* Daughterless and the E proteins E12 and E47 which form heterodimers with Class B bHLH proteins for DNA binding. Examples of class B proteins are Neurogenin1 (Neurog1), Neurogenin2 (Neurog2), Ascl1 (Mash1), Atoh1 (Math1), and NeuroD (Beta2).

bHLH genes function in regulatory cascades during neurogenesis and have been categorized based on their expression pattern and timing of function into two groups: determination genes and differentiation genes. Determination genes are expressed by many neuronal precursors and regulate the acquisition of general pan-neuronal properties. Differentiation genes are expressed more specifically in subsets of early differentiating

neurons and are involved in the acquisition of specific neuronal identities (Guillemot, 1999). This distinction is more functional than absolute, however. Pan-neuronal determination bHLH genes include *Neurog1/2*, *Ascl1*, and *Math1*, but these genes have been shown to confer some aspects of neuronal specification (Cai et al., 2000). *NeuroD* and *Nscl* genes are involved in differentiation downstream of neuronal determination bHLH factors, but play a role in cell fate acquisition as well (Fode et al., 1998; Ma et al., 1998; Guillemot, 1999).

A third dimension used to classify groups of bHLH genes is based on their mode of function: repressor type and activator type (Kageyama et al., 2005). Repressor type bHLH genes include *Hes* and *Id* genes. *Hes1* and *Hes5* are widely expressed in neural progenitor cells and repress proneural bHLH genes, thus maintaining neural progenitor populations. For example, Hes1 can repress *Atoh1* or *Neurog2* expression by forming a complex with the corepressor TLE/Groucho and repressing transcription by binding to the promoters of these genes (Chen et al., 1997; Kageyama et al., 2005). Hes1 also binds to the ubiquitous bHLH cofactor E47 and interferes with the ability of activator bHLH proteins such as Atoh1 to form functional heterodimers needed for activity (Sasai et al., 1992; Kageyama et al., 2005). The bHLH Id proteins lack a functional DNA binding domain and heterodimerize with other bHLH factors to act in a dominant negative fashion, inhibiting their ability to activate transcription (Peyton et al., 1996).

Combinations of these dimensions have been used to distinguish functional families of bHLH genes, defined on the basis of DNA binding specificity, tissue specific expression, the ability to form homo- or heterodimers, and transcriptional activity (Fig. 1.3; Murre et al., 1994; Massari and Murre, 2000). The *asc* family includes *ash1*

(*Ascl1*/*Mash1* in mouse, *Cash1* in chicken) and *Mash2*. Vertebrate relatives of *ato* have been grouped into a number of subfamilies based on sequence similarity, primarily in the bHLH domain. *Ato* genes include *Math1*, *Math5*, and several related subfamilies, including the neurogenin family (*Neurog*, including *Neurog1* and *Neurog2*), the *NeuroD* family (including *NeuroD*, *NeuroD2*, and *Math2*), and the *Olig* family (including *Olig1*, *Olig2*, *Olig3*, *Bhlhb4*, and *Bhlhb5*). These genes have diverse and important functions in neuronal development, including regulation of neural progenitor formation, neuronal differentiation, and cell cycle exit.

In vertebrates, bHLH genes function in many stages of neuronal development. Some have proneural functions similar to their *Drosophila* counterparts, such as genes of the *asc* family, whereas others are involved in differentiation or specification of neuronal fates but not in progenitor selection (Bertrand et al., 2002). bHLH proneural genes perform their functions by activating transcriptional factor regulatory cascades (Mattar et al., 2004). Proneural genes are activated in neural precursors, are downregulated as progenitors exit the cell cycle and differentiate, and themselves activate downstream determination genes that are expressed in immature neurons. For example, although both *Neurog* and *NeuroD* can activate neurogenesis when overexpressed, *Neurog* is expressed transiently in committed neural progenitors in the ventricular zone whereas *NeuroD* expression follows during differentiation (Sommer et al., 1996). *Neurog* acts upstream of *NeuroD* but both can activate similar target genes, suggesting that *Neurog* expression initiates a program of neurogenesis that is sustained by *NeuroD* in differentiating cells (Seo et al., 2007).

This transcriptional cascade couples selection of neuronal progenitors with differentiation into neurons. In motor neuron formation, *Olig2* expression establishes the progenitor domain and induces expression of (a) the pan-neuronal bHLH protein Neurog2, (b) the downstream proneural differentiation gene *NeuroD4* and (c) the motor neuron-specific transcription factors *Lhx3* and *Isl1/2* which then cooperate to activate transcriptional targets characteristic of mature motor neurons (Lee and Pfaff, 2001; Lee et al., 2005).

In ventral spinal cord, *Olig2* is required for establishment of the motor neuron progenitor domain, although HD proteins establish all other ventral progenitor domains and Nkx6 proteins regulate initial *Olig2* expression (Novitch et al., 2001). In dorsal spinal cord, there are six progenitor domains that give rise to discrete populations of neurons, labeled dI1 through dI6 from dorsal to ventral (Fig. 1.1B; Caspary and Anderson, 2003; Helms and Johnson, 2003). Unlike in ventral spinal cord, there are no unique combinations of HD factors that mark individual progenitor domains, although HD factors are expressed in these cells. *Math1*, *Neurog1*, and *Ascl1* are expressed in mutually exclusive progenitor domains where they generate distinct subtypes of mature interneurons (Caspary and Anderson, 2003). In dorsal spinal cord, signals from the roof plate establish progenitor domains through bHLH gene expression. *Math1* is expressed in the most dorsal domain which gives rise to dI1 interneurons, *Neurog1* is expressed in the adjacent dI2 domain, and *Ascl1* is expressed in the intermediate dorsal domains that will give rise to dI3-dI5 interneurons. *Neurog2* expression partially overlaps with *Neurog1* and *Ascl1* and is more broadly expressed in progenitor domains of the ventral neural tube (Helms et al., 2005). Sharp boundaries between these progenitor domains are

maintained through cross-repressive interactions among bHLH factors (Gowan et al., 2001) similar to ventral progenitor domain boundary maintenance by cross-repression between HD proteins.

These bHLH genes orchestrate both proneural and subtype specification in dorsally derived neuronal types. In *Math1* mutants, dI1 interneurons fail to form and dI2 interneurons are produced in increased numbers. Conversely, misexpression of *Math1* can produce ectopic dI1 interneurons at the expense of other types (Bermingham et al., 2001; Nakada et al., 2004). Similarly, in *Neurog1;Neurog2* double mutants, dI2 interneurons are absent and *Math1* expression expands ventrally (Gowan et al., 2001). *Ascl1* marks progenitors in the dI3-dI5 domains and appears to have a complex role in cell type specification in dorsal spinal cord, in contrast to its more straightforward function in specification of GABAergic neurons in the developing telencephalon (Fode et al., 2000; Parras et al., 2002; Helms et al., 2005; Müller et al., 2005).

Ascl1 is expressed in the progenitor populations that give rise to dI3-dI5 neurons, and can drive generation of dI3 and dI5 populations but not dI4. In *Ascl1* null embryos, dI3 and dI5 neurons fail to form and there is an increase in dI2 and dI4 neurons; similarly, *Ascl1* misexpression increases the numbers of dI3 and dI5 subtypes at the expense of dI2 and dI4 neurons (Helms et al., 2005). In contrast, *Neurog2* is not required for formation of any of the individual neuronal subtypes but does have a role in limiting the generation of dI3 and dI5 subtypes downstream of *Ascl1*. Surprisingly, although neither *Mash1* nor *Neurog2* is required for generation of dI4 neurons, in the absence of both of these factors, dI4 neurons fail to form (Helms et al., 2005). Another bHLH factor expressed in postmitotic cells, *Ptf1a*, is found in dI4 neurons whose progenitors express

low levels of *Ascl1* (Glasgow et al., 2005; Henke et al., 2009). *Ptf1a* is required for generation of the dI4 population while repressing dI5 fates. In addition, the proneural *Neurog2* is a direct target of *Ptf1a* transcriptional activity (Henke et al., 2009). These results illustrate the complex and domain-specific interactions among bHLH and HD proteins in various spatial and temporal aspects of neuronal production in the dorsal spinal cord.

Complex interactions exist among bHLH and HD transcription factors in specifying cell fates within the dorsal spinal cord. In dI4-dI6 domains, *Ascl1* promotes expression of the HD factor *Lbx1* as the fate specifying protein for postmitotic dI4-dI6 neurons. In *Lbx1* mutants, dI4 and dI5 interneurons are lost; instead these neurons acquire more dorsal dI2-dI3 fates and misexpression has the converse effect (Müller et al., 2002). Interestingly, the bHLH transcription factor *Olig3* is coexpressed with *Math1*, *Neurog1/2*, and *Ascl1* in dI1, dI2, and dI3 progenitor domains, respectively, in the dorsal spinal cord (Müller et al., 2005). In *Olig3* mutants, numbers of dI1 neurons are reduced and dI2-dI3 neurons are completely lost, acquiring characteristics of dI4 neuron types (Müller et al., 2005). Similar effects are seen with manipulation of *Lbx1* (Müller et al., 2002; Müller et al., 2005) but the loss of dI2 and dI3 neurons in this case occurs through a different mechanism. *Olig3* does not exhibit strong proneural effects on its own, but cooperates instructively with *Ascl1* in specification of dI3 neurons. In contrast, generation of dI2 neurons by *Olig3* is attributed to direct suppression of *Lbx1* expression rather than direct specification of dI2 cell fates (Müller et al., 2005).

In ventral spinal cord, with the exception of the pMN domain defined by expression of *Olig2*, progenitor domains are defined by combinatorial expression of HD

proteins. However, bHLH factors play important roles in acquisition of specific cell fates in ventral as well as dorsal spinal cord and they participate in cross-regulatory interactions with HD proteins to do so. For example, the proneural bHLH gene *Neurog2* is expressed along the dorsal-ventral extent of the developing neural tube. However, *Neurog2* expression is controlled by different enhancer elements in distinct progenitor domains and are differentially regulated by the HD protein Pax6 depending on the dorsoventral position of the domain (Scardigli et al., 2001). Interestingly, *Neurog2* is reciprocally involved in maintenance of *Pax6* and *Nkx2.2* expression, suggesting intersecting regulatory feedback loops between HD and bHLH proteins in progenitor patterning, subtype specification and balance between proliferation and differentiation. Partial redundancy between multiple bHLH factors is also observed in ventral spinal cord. For example, neuronal production is retained in *Neurog2* mutants due to compensation for its proneural effects by *Neurog1* in the same domains (Scardigli et al., 2001). However, *Neurog1* is unable to compensate for *Neurog2* function in the regulation of HD gene expression (Scardigli et al., 2001).

bHLH genes play essential roles in complex ways in promoting neurogenesis and influencing cell type specification in the developing neural tube. There must be balance between maintenance of a progenitor pool and the creation of particular types of neurons in specific places at defined times in order to establish all of the cell types and interconnections that must be made for the CNS to function. Stringent control of the numbers and types of neurons created must be exerted throughout development. Another component for controlling the timing and place of neuronal differentiation involves the Notch signaling pathway, a pathway that is intimately linked to bHLH gene function.

Notch signaling in development

Notch signaling was first described as an important mechanism regulating neurogenesis in *Drosophila* (Poulson, 1940). The basic process of canonical Notch signaling is conserved across many species (Louvi and Artavanis-Tsaknonas, 2006). The extracellular domain of one of the transmembrane Notch ligands, Delta or Jagged (known as Serrate in flies and some vertebrate species), resides on one cell and interacts with the extracellular domain of the transmembrane Notch receptor on a neighboring cell (Fig. 1.4). Activation of the Notch receptor results in proteolytic cleavage of an intracellular domain of the Notch receptor (NICD) that releases it from the membrane and allows it to translocate into the nucleus. In the nucleus, NICD forms a complex with DNA-binding cofactors such as CSL to activate transcription of the bHLH transcriptional repressors such as *Hes* and *Hey* genes. *Hes1* and *Hes5* are direct targets of Notch activation in many neural progenitors and their repressor function serves to maintain cells in a progenitor state through repressing transcription of proneural bHLH target genes such as *Neurogs*. Notch is best known for its proneural role (Jan and Jan, 1993) but it also plays a role in patterning of progenitor domains, establishment of boundaries, and cell fate specification.

A central concept emerging from studies of Notch signaling in *Drosophila* neuroblast development is the notion of lateral inhibition. Lateral inhibition limits the proportion of neural progenitor cells that adopt distinct neural or non-neural fates within a homogeneous progenitor population. Initially equivalent cells each express comparable levels of Notch receptor and ligands. Over time, small differences in Notch receptor signaling arise between cells through stochastic variations or dynamic changes in gene

expression (Kageyama et al., 2008). These small differences become amplified through transcriptional feedback loops. Notch activation upregulates *Hes* genes, which in turn repress proneural bHLH genes in the activated cell, maintaining the cell in an undifferentiated state. Notch receptor and ligands are also directly regulated by Notch activation. Over time, this leads one cell to express high levels of Notch receptor and low levels of ligand, such that it becomes a "receiving" cell, and its neighbor to express low levels of Notch receptor and high levels of ligand, a "sending" cell, as well as initiating proneural gene activity in the sending cell. In this way, proneural gene expression in an initially equivalent group of neural progenitor cells becomes restricted to a single cell which differentiates as a neuron while preventing neighboring cells from doing so (Bertrand et al., 2002; Fortini, 2009).

There is increasing evidence for regulatory interactions among cell-fate determining transcription factors, proneural bHLH proteins, and signaling pathways such as Notch (Marklund et al., 2010). Notch signaling interacts with other signaling systems, positional cues and patterning genes to coordinate distribution of different cell types within a tissue. The coupling of Notch signaling to acquisition of particular subtype identities is one way that specification of neuronal cell types in the correct numbers and at the correct time can be linked. For example, in motor neuron progenitors, *Olig2* influences the balance between progenitor maintenance and differentiation through regulation of expression of the proneural bHLH protein Neurog2 (Novitch et al., 2001). Conversely, Notch downstream target genes, such as Hes1 and Hes5, function through repression of proneural proteins such as Neurog2 (Louvi and Artavanis-Tsakonas, 2006). Furthermore, both *Olig2* and Notch cooperate in the switch from the generation of

neurons to the generation of oligodendrocytes from the same progenitor domain. *Olig2* and Notch thus provide coordinate inputs to control neurogenesis in the motor neuron progenitor domain and cooperate in regulating timing and cell fate choices within that domain.

In the developing spinal cord, particular Notch ligands are expressed in discrete progenitor domains (Lindsell et al., 1996; Myat et al., 1996; Rocha et al., 2009; Marklund et al., 2010). The Notch ligands Dll1 and Jag1 are expressed in a complementary pattern throughout the spinal cord. Expression of both *Dll1* and *Jag1* is confined to specific progenitor domains and a recent study has demonstrated that their expression is controlled by the HD proteins that establish those domains (Marklund et al., 2010). In *Dll1* and *Jag1* mutants, correct specification of progenitor domains is maintained and effects on neurogenesis are confined to those domains that normally express the particular ligand. Loss of *Jag1* leads to an increase in production of V1 neurons that derive from the p1 domain but has no effect on the number of V0, V2, or motor neurons produced from other domains (Marklund et al., 2010). Loss of *Dll1* has the opposite effect, increasing the number of V0, V2, and motor neurons produced but having no effect on the number of V1 neurons (Rocha et al., 2009; Marklund et al., 2010). Thus, in the developing spinal cord, neurogenesis within specific progenitor domains is controlled independently through differential distribution of Notch ligands, endowing distinct progenitor domains with local regulatory control of the timing and pace of neurogenesis. However, despite distinct domains of ligand expression, the regulation of the bHLH downstream effector elements of Notch signaling, such as *Hes5* and *Neurog2*, appear to

be controlled through canonical Notch receptor-ligand interactions (Marklund et al., 2010).

The Notch signaling pathway has a major regulatory role in neurogenesis, primarily by promoting maintenance of progenitor cell populations, (Louvi and Artavanis-Tsakonas, 2006; Kageyama et al., 2008; Marklund et al., 2010) but also affects neuronal subtype specification. This has been most clearly demonstrated in the generation of discrete neuronal subtypes of V2 neurons in ventral spinal cord, where Notch signaling mediates the progression of neurogenesis in the p2 progenitor domain as well as the choice between V2a and V2b neuronal fates (Del Barrio et al., 2007; Peng et al., 2007). In the developing spinal cord, the Notch ligands Dll1 and Dll4 are both expressed in the p2 domain. *Dll1* is expressed throughout the dorsal-ventral extent of the neural tube (except in areas of *Jag1* expression), including in p2 progenitors. *Dll4* expression, on the other hand, is restricted to the p2 domain (Benedito and Duarte, 2005; Peng et al., 2007; Rocha et al., 2009; Marklund et al., 2010). Temporal analysis of *Dll1* and *Dll4* expression indicates that *Dll1* is expressed earlier than *Dll4* in p2, although *Dll4* continues be expressed in *Dll1* mutants as is the Notch target gene *Hes5*. This indicates that *Dll1* and *Dll4* are generated independently and that both can play a role in activation of Notch and maintenance of the progenitor cell population (Rocha et al., 2009). In the absence of Notch signaling, massive increases in neurogenesis within the p2 domain produce increased numbers of V2 neurons but all of them differentiate into the V2a subtype at the expense of the V2b cell fate (Yang et al., 2006; Del Barrio et al., 2007; Peng et al., 2007) showing lack of proper cell fate decisions in the absence of Notch.

In *Dll1* mutants, which still express *Dll4* in the p2 domain, both V2a and V2b neurons are produced, with an increase in numbers of V2a but equivalent numbers of V2b subtypes compared to controls (Rocha et al., 2009). Misexpression of *Dll4* results in an increase in V2b neurons that is accompanied by a decrease in the number of neurons adopting a V2a fate whereas misexpression of *Dll1* does not significantly alter the number of V2a and V2b neurons produced (Del Barrio et al., 2007; Peng et al., 2007). These results indicate that while Notch-Dll1 signaling regulates the general balance between progenitor maintenance and differentiation, Notch signaling through Dll4 regulates the acquisition of distinct fates in a V2a-V2b binary fate choice (Yang et al., 2006; Del Barrio et al., 2007; Peng et al., 2007; Rocha et al., 2009).

A direct relationship between bHLH gene function and Notch signaling has been demonstrated in multiple tissues during vertebrate neural development. *Ascl1* is expressed in a number of tissues in the peripheral and central nervous system. In the ventral telencephalon, loss of *Ascl1* proneural function results in the absence of neuronal precursor cells specifically in the medial ganglionic eminence (Casarosa et al., 1999). However, in other areas of the ventral telencephalon, mesencephalon and dorsal spinal cord, neuronal precursors are specified correctly in *Ascl1* mutants while expression of the Notch ligands Dll1 and Dll4 and the Notch target gene *Hes5* is lost (Casarosa et al., 1999). Thus, *Ascl1* is required for Notch signaling in the ventral telencephalon and dorsal spinal cord and its absence leads to premature acquisition of differentiation markers and loss of neuronal progenitors, independent of its role as a neuronal determinant (Casarosa et al., 1999). Interestingly, misexpression of *Ascl1* in the ventral spinal cord promotes expression of the Notch ligand Dll4 and inhibits expression of *Dll1*

in V2 neuronal progenitors. This increases the number of neurons adopting a V2b fate at the expense of V2a fates, although *Dll4* expression, which can be induced by *Ascl1*, is normal in *Ascl1* mutants (Del Barrio et al., 2007; Peng et al., 2007). This implicates the bHLH protein Ascl1 as sufficient, but not required, for linking Notch signaling to specification of particular neuronal subtypes in this area of the developing neural tube. Whether a related proneural protein such as Neurog2 might have a compensatory effect on *Dll4* expression in the absence of *Ascl1* is unknown (Del Barrio et al., 2007).

It has been hypothesized that differences in the function of proneural bHLH genes (such as between *asc* family member *Ascl1* and *ato*-related *Neurogs* or between proneural determination and differentiation genes like *Neurog* and *NeuroD*) in promoting neurogenesis might be attributed to differential sensitivities to Notch signaling (Bertrand et al., 2002). Differential Notch sensitivity has been observed in other species; for example, in Xenopus, *Xash3* and *NeuroD*, which are sequentially expressed as they are in mammals, both have proneural activity but induce ectopic neuron formation in different domains, *Xash3* in the neural plate and *NeuroD* in the neural plate and in epidermis (Chitnis and Kintner, 1996; Lo et al., 2002). *Xash3* and *NeuroD* also promote expression of the Notch ligand X-Delta1. However, only the *Xash3* proneural activity is inhibited by X-Delta1-activated Notch or by injections of the activated Notch intracellular domain; *NeuroD* is relatively insensitive to inhibitory effects of activated Notch and thus promotes ectopic neuron formation in a much wider domain. These effects are consistent with the differential roles of *Ascl1* and *NeuroD* in progenitor as opposed to differentiation domains. In cell culture experiments, the ability of Ascl1 to promote neuronal

differentiation has been observed to be much more sensitive to Notch-mediated inhibition than that promoted by *Neurog1* (Lo et al., 2002).

This differential sensitivity to inhibition of proneural activity is suggested to underlie lineage-specific distribution of *Ascl1* and *Neurogs* in neural progenitors. For example, in the developing telencephalon, *Neurogs* are expressed in cortical progenitors that migrate and differentiate shortly after leaving the ventricular zone, exhibiting relatively rapid differentiation. In contrast, *Ascl1* is expressed in ventral progenitors that are generated more slowly and migrate longer distances before differentiating, requiring more extended periods of progenitor maintenance (Fode et al., 2000; Lo et al., 2002). Differential sensitivity to patterning morphogens, such as concentrations of Bmps secreted by the roof plate during spinal neurogenesis, is also related to the dorsal-ventral distribution of proneural genes during development.

Another method of regulation of Notch signaling is also through posttranslational modification of ligands and receptors that modulate their responsiveness to signaling. These posttranslational modifications primarily involve differential glycosylation of receptor and ligand which is carried out by Fringe (Fng) proteins (Bray, 2006). Both Notch ligands and Fng proteins have distinct patterns of distribution, which modulate Notch responsiveness in a domain-dependent manner. In the developing neural tube, expression of the Notch modulating Fng proteins is also controlled by HD patterning proteins specific to particular progenitor domains domains, which are responsible for the domain restricted expression of Notch ligands (Marklund et al., 2010). Notch and Fng interact differently in different tissues so coordinate regulation of their expression can modulate Notch signaling in a tissue-dependent fashion. In the developing spinal cord,

Fng and *Dll1* are expressed in the same domains whereas Fng proteins are excluded from progenitor domains that express *Jag1*. Manipulation of the expression of Fng proteins in the developing spinal cord independent of their normal ligand partner indicates that they indeed influence the response to Notch signaling within particular domains (Marklund et al., 2010). Overexpression of *Fng* enhances Notch signaling within the MN and V0 domains, where it is normally coexpressed with *Dll1*, and reduces the production of mature neurons by 10-20%. In contrast, production of V1 neurons from the *Jag1*-expressing p1 domain is increased by 160%. This dramatic difference in responsiveness indicates that Fng can augment Notch signaling within $Dll1^{+}$ domains and lead to a modest increase in progenitors. On the other hand, obstruction of Notch signaling in $Jag1^{+}$ domains by Fng leads to a much greater differentiation response (Marklund et al., 2010).

Identification of Bhlhb5 as a candidate gene in regulation of spinal cord development

In order for the bHLH transcriptional repressor *Olig2* to drive expression of motor neuron-specific and pan-neuronal differentiation markers, it must regulate expression of other regulatory genes that regulate progenitor cell maintenance, direct cell-cycle exit, influence differentiation, or direct the development of alternate neuronal fates. A comparison of genes expressed in $Olig2^{+}$ vs $Olig2^{-}$ ventral spinal cords was undertaken using $Olig2^{GFP}$ knock-in mice that allowed the isolation of MN progenitors using fluorescence activated cell sorting (FACS) (Rousso et al., 2008). A number of genes were identified that exhibited either broad expression patterns in the ventricular zone of the developing neural tube indicative of progenitor domains or restricted expression

patterns suggestive of their involvement in subsets of progenitors or differentiated interneuron classes. One of the most interesting genes of the latter group was *Bhlhb5*, a bHLH transcription factor highly related to *Olig2* in structure (Fig. 1.5). *Bhlhb5* had been shown to be expressed in brain and spinal cord but its function in these tissues had not been investigated (Xu et al., 2002; Brunelli et al., 2003). Based on its expression pattern in embryonic mouse spinal cord, *Bhlhb5* appeared to be expressed in several populations of ventral interneurons and their progenitors adjacent to where motor neurons are formed. In *Olig2* null spinal cords, Bhlhb5 expression expanded ventrally to encompass the progenitor area normally occupied by Olig2 and was accompanied by an expansion in interneuron subtype markers (Novitch et al., 2001; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002), suggesting that *Bhlhb5* might have a role in specification of interneuron identity analogous to that of *Olig2* in the formation of motor neurons.

Bhlhb5

Bhlhb5 is a member of a large family of bHLH transcription factors related to the *Drosophila atonal* gene (Fig. 1.3). Members of this group include several genes crucial to neural development, including the *NeuroD* and *Neurogenin* subfamilies. *Bhlhb5* belongs to a bHLH subfamily that includes the closely related genes *Olig1*, *Olig2*, *Olig3*, and *Bhlhb4* (Bramblett et al., 2002). Many of these proteins act as negative regulators of the function or transcription of other bHLH proteins, as do the *Id* and *Hes* bHLH families.

Bhlhb5 was first described in a screen for novel bHLH proteins in a hamster insulin tumor cell line (Peyton et al., 1996). Tissue distribution assays found highest levels of Bhlhb5 expression in brain, kidney and lung (Peyton et al., 1996). Bhlhb5 interacts with E proteins and represses the activity of other bHLH proteins (Peyton et al., 1996). Early studies using cell culture transfection assays demonstrated that, in contrast to NeuroD, Bhlhb5 does not bind an insulin E box, but represses NeuroD DNA binding and transcription of a reporter construct driven by the same E box (Peyton et al., 1996). Bhlhb5 repression of Pax6 transcription is dependent on a promoter containing a consensus E box, but that repression of reporter activity still occurs, albeit at a lower level, when the basic DNA-binding domain is deleted (Xu et al., 2002). Other bHLH factors, such as MyoD and NeuroD, were not able to bind to or repress reporter activity from the Pax6 promoter (Xu et al., 2002). This led to the suggestion that Bhlhb5 exerts its action through a non-DNA binding mechanism, possibly through heterodimerization and sequestration of class A bHLH factors similar to Id proteins. This is surprising, since *Bhlhb5* contains a complete basic DNA-binding domain in all species examined (Peyton et al., 1996; Xu et al., 2002).

Bhlhb5 expression in mouse is first detected around E9.0 in brain and in two longitudinal columns in the caudal neural tube (Brunelli et al., 2003). Its expression becomes more widespread in forebrain, midbrain, and hindbrain and in the otic placode by E10.5. Expression in the mouse CNS continues throughout embryonic development, becoming quite prominent by E13 in specific layers of the cerebral cortex, piriform cortex, and midbrain, particularly the cortical plate and subventricular zone. *Bhlhb5* is expressed in the developing hippocampus by E17 with especially strong expression in the

dentate gyrus and CA layers (Kim et al., 2002) which persists into adulthood. *Bhlhb5* is also expressed in the developing cerebellum in the Purkinje cell layer and in granule cells (Kim et al., 2002). In addition to CNS expression, *Bhlhb5* is strongly expressed in various sensory organs (inner and outer retinal layers, inner ear and sensory layer of the cochlea, olfactory epithelium) and hair follicles (Kim et al., 2002; Brunelli et al., 2003). Given the role of bHLH factors in neurogenesis, patterning and cell fate specification in the developing CNS, the localized expression of *Bhlhb5* in discrete regions during development, as well as its overlap with other known neurogenic factors, suggests that *Bhlhb5* might play critical roles in neuronal cell-type specification and differentiation.

Experimental analysis of Bhlhb5 function

Bhlhb5 regulates specification of some amacrine and bipolar cell types in retina

The first analysis of Bhlhb5 function explored its role in cell fate specification in the retina (Feng et al., 2006). The retina consists of a well-defined laminar structure of six major neuronal cell types (retinal ganglion cells, rod and cone photoreceptors, and bipolar, horizontal, and amacrine interneurons) and one type of Müller glia. Retina is a frequently used system for the study of transcription factors and their roles and interactions in cell fate determination, including many of the transcription factors known to play a role in spinal cord neurogenesis. For example, the HD protein Chx10 and the bHLH proteins Ascl1 and Neurod4 (Mash1 and Math3) specify bipolar fates. In the absence of *Chx10* or *Ascl1* and *Neurod4*, bipolar cells fail to develop. Misexpression of any of the three factors *Chx10*, *Ascl1*, or *Neurod4* alone fails to induce bipolar cells, whereas misexpression of *Chx10* plus *Ascl1* or *Neurod4* increases the number of bipolar

cells generated (Hatakeyama et al., 2001). Similarly, the homeobox transcription factor *Pax6* and the bHLH factors *Math3* and *Neurod1* cooperate in the specification of amacrine cells (Inoue et al., 2002).

Bhlhb5 expression in mouse retina begins at E11.5 in the neuroblast layer and becomes progressively localized in the mature retina to select groups of cells in two rows in the inner nuclear layer and one row in the granule cell layer (Feng et al., 2006). Coexpression with markers of specific retinal cell subtypes indicates that the *Bhlhb5* is expressed in *Pax6*$^{+}$ amacrine cells in the inner nuclear layer and the ganglion cell layer and that the majority of these are of the GABAergic subtype. At the outer boundary of the inner nuclear layer, *Bhlhb5*$^{+}$ cells coexpress the bipolar cell marker *Chx10* and the cone-bipolar specific marker *Vsx1*. Interestingly, *Vsx1*$^{-}$ rod bipolar cells express the highly related bHLH transcription factor *Bhlhb4*, indicating that these two very similar family members direct two different bipolar cell fates.

The *Bhlhb5*-expressing cells are a subset of Type 2 OFF cone bipolar cells as determined through colocalization with the protein Recoverin, representing about 23% of the total population. Thus, *Bhlhb5* identifies two different neuronal populations in the mature retina (Feng et al., 2006). Investigation of retinal cell neurogenesis in *Bhlhb5*-null mutant mice indicates a 35-50% reduction in the number of amacrine and cone bipolar cells that normally express *Bhlhb5* in the mature eye. The number of cells in the neuroblast layer expressing early retinogenic factors such as *Neurod1*, *Math2*, and *Ascl1* is unchanged in the embryonic *Bhlhb5* mutant eye, suggesting that *Bhlhb5* influences subtype specification in differentiated post-mitotic retinal neurons rather than initial progenitor character. Intriguingly, loss of *Bhlhb5* also results in a reduction in

dopaminergic amacrine cells that do not express *Bhlhb5* and there is no compensatory increase in other cell types. There are several possible explanations for these results: (a) expression of *Bhlhb5* is transient in the dopaminergic amacrine cell lineage, (b) proliferation is reduced in the absence of *Bhlhb5* but goes undetected due to the small percentage of *Bhlhb5*$^{+}$ cells, or (c) interaction of *Bhlhb5* with other bHLH factors fine-tunes the specification programs (Feng et al., 2006). Further investigation is necessary to determine the precise mechanism of *Bhlhb5* influence on retinal cell subtype specification.

Bhlhb5 regulates area-specific identities and neuronal migration in the cortex and corticospinal tracts

Bhlhb5 function in the developing cortex has also been recently examined in detail (Joshi et al., 2008). In agreement with earlier descriptive accounts, *Bhlhb5* expression is most pronounced in the cortical plate between E13.5 and E17.5 in the developing mouse brain. Some Bhlhb5 staining is detected in the subventricular zone but *Bhlhb5* expression is restricted to postmitotic neurons, as it is in retina (Feng et al., 2006). These neurons are presumptive corticospinal motor neurons (CSMN) and other subcerebral projection neurons in the developing cortical layer V as shown by costaining with CTIP2, a transcription factor that labels all subcerebral projection neurons. *Bhlhb5* expression is observed in layers superficial to the *CTIP2*-expressing layers. Initially expressed in a high caudomedial to a low rostrolateral gradient, *Bhlhb5* expression becomes increasingly refined to demarcate a sharp boundary between rostral motor and sensory domains. By early postnatal timepoints, *Bhlhb5* is expressed selectively in the

primary visual cortex, primary auditory cortex, and primary somatosensory cortex, thus defining distinct areas of the cortical map in layer IV (Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002; O'Leary et al., 2007).

Cortical layers are morphologically normal in *Bhlhb5*-null mice, although layer V in the sensorimotor cortex is compacted, with a disruption of sublayer organization. Several disruptions in gene expression patterns are present in the somatosensory cortex of *Bhlhb5* nulls: ectopic expression of *Cadherin 8* in layers II-III from which it is normally absent, lack of expression of the orphan nuclear receptor *COUP-TF1*, decreased expression of the axon guidance molecule *Eprin-A5* in layers IV-V, and loss of the layer IV transcriptional regulator Lmo4. Selective and contrasting defects along the mediolateral axis are also observed in the cortex of *Bhlhb5*-null mice. The layer IV orphan nuclear receptor RORß is largely unchanged laterally in the somatosensory cortex but is not expressed medially in the caudal motor cortex of *Bhlhb5* nulls. Similarly, the bHLH factor *Id2*, which marks the border between sensory and rostral motor cortex in layer V, is expressed in the somatosensory cortex but lost in the caudal motor cortex in *Bhlhb5* nulls. These disruptions in gene expression are not accompanied by positional shifts of cortical regions as there is no change in the ratio of rostral motor to sensory domain surface areas in the mutants. These results suggest that *Bhlhb5* is not involved in specification or establishment of general regional identities but in the acquisition of area-specific neuronal subtypes.

Projection neuron subtypes affected in *Bhlhb5* null mice include two types of subcerebral projection neurons: CSMN, which send projections to spinal cord, and corticotectal projection neurons (CTPN), which send projections to the midbrain superior

colliculus. *Bhlhb5* is expressed in CSMN in caudal but not rostral motor cortex and in presumptive CTPN in the occipital cortex. Gene expression in occipital cortex is mostly unaltered in *Bhlhb5* nulls and CTPN tracts are also preserved. Consistent with disruption of gene expression in caudal motor cortex, however, CSMN projections from caudal motor cortex are severely disrupted in *Bhlhb5* nulls. CSMN axons descend from the motor cortex and project through the internal capsule, then through the cerebral peduncle into the midbrain and pons and through the pyramidal tract in the ventral medulla, where they cross and descend into the spinal cord to settle in the sensory dorsal funiculus (Koester and O'Leary, 1994; Molyneaux et al., 2007). The size of the medullary pyramidal tract in adult *Bhlhb5* nulls is severely reduced compared to controls, suggesting that these projections are lost. Axon tracing showed that CSMN projections in *Bhlhb5* nulls appear normal in the internal capsule and cerebral peduncle but do not enter the pyramidal tract and thus fail to extend to their spinal cord targets. Interestingly, although *Bhlhb5* is not expressed in rostral CSMN, axons from these cells also fail to enter the corticospinal tract in *Bhlhb5* mutant mice (Joshi et al., 2008).

In summary, extensive analysis of *Bhlhb5* function in the developing cortex has demonstrated that *Bhlhb5* is critically involved in acquisition of specific neuronal identities in layers II-V. The initial gradient of *Bhlhb5* expression becomes a definitive marker of the border between sensory and rostral motor cortex. Layer-specific cell identities are disrupted and CSMN neurons fail to form correctly in *Bhlhb5* null mice. *Bhlhb5* functions in the acquisition of area-specific properties in postmitotic neurons of the somatosensory and motor cortex, subsequent to the initial establishment of rostrocaudal and mediolateral positional identity (Joshi et al., 2008).

Bhlhb5 regulates itch response in dorsal horn inhibitory interneurons

Despite the loss of the CSMNs and reduction of the corticospinal tract, motor behavior in *Bhlhb5* null mice is grossly normal, although they do exhibit tremor when suspended and sometimes shuffle and clasp their forelimbs (Joshi et al., 2008). In addition, *Bhlhb5* null mice develop skin lesions, unlike their wildtype or heterozygous littermates. The circuits that mediate itch and pain are poorly understood. Recent evidence suggests that they are mediated by distinct sets of neurons within the dorsal horn of the spinal cord (Sun et al., 2009). *Bhlhb5* is expressed in a subset of late-born interneurons, designated dIL neurons, that settle in the uppermost laminae of the dorsal horn in an area that may be involved in transmission of pain and itch signals (Liu et al., 2007).

The functional significance of this dorsal *Bhlhb5*$^{+}$ group of cells was recently examined (Ross et al., 2010). Mice lacking *Bhlhb5* develop skin lesions by 4-8 weeks of age due to excessive licking and scratching. These mice were found to be hyperresponsive to itch-inducing agents but no different from controls in their response to pain (Ross et al., 2010). Itch and pain signals are mediated by specific peripheral DRG neurons, but innervation by these neurons is unchanged in *Bhlhb5* mutants. Lineage tracing of dorsal horn *Bhlhb5* neurons using a *Bhlhb5*-cre mouse line to drive expression of a reporter construct showed that these responses are mediated by *Bhlhb5*-expressing neurons in the dorsal horn of the spinal cord. Although no gross disruptions of laminae or architecture of the dorsal horn were observed in *Bhlhb5* nulls, lineage tracing revealed a 50% reduction in the number of neurons that would have expressed *Bhlhb5* in the

superficial laminae of the dorsal horn in *Bhlhb5*$^{cre/-}$ mice compared to *Bhlhb5*$^{cre/+}$ animals (Ross et al., 2010). In the dorsal spinal cord, *Bhlhb5* is expressed postmitotically in a late born population of interneurons that settle in the superficial layers of the dorsal horn (Liu et al., 2007). Despite comprising only a fraction of dorsal horn interneurons, these *Bhlhb5*$^{+}$ neurons represent a heterogeneous population with some cells coexpressing markers of inhibitory dIL^{A} and others coexpressing markers of excitatory dIL^{B} subtypes (Liu et al., 2004; Ross et al., 2010). In *Bhlhb5* mutant spinal cords, similar proportions of both subtypes are lost (Ross et al., 2010). Conditional knockout of *Bhlhb5* in only inhibitory (using *Pax2*-cre) or only excitatory (using *Tlx3*-cre) neurons revealed that it is loss of inhibitory neurons that leads to the heightened itch response in *Bhlhb5* null mice. Interestingly, cell loss in the dorsal horn of *Bhlhb5* nulls is not due to aberrant migration as seen in cortex (Joshi et al., 2008), but is accompanied by an increase in apoptotic cell death at E18.5 in the superficial dorsal horn, suggesting that *Bhlhb5* is required for survival of these neurons (Ross et al., 2010).

BHLHB5 in human disease states

In humans, *BHLHB5* maps to chromosome 8q13 (Xu et al., 2002). It is 94% identical to mouse *Bhlhb5* at the protein level and 89% homologous at the level of cDNA. Human and mouse genes have identical bHLH domains and contain proline-rich and glycine-rich regions. In humans, *BHLHB5* is highly expressed in neural tissues, including cerebral cortex, cerebellum, and occipital pole. However, whereas mouse *Bhlhb5* mRNA is detected on Northern blots as a single band of approximately 3.6 kb, there are two apparent isoforms of human *BHLHB5* that yield transcripts of 3.8 and 3.0

kb respectively in all tissues examined (Peyton et al. 1996; Kim et al., 2002; Xu et al., 2002). Examination of the promoter region of human *BHLHB5* found two predicted promoter sequences upstream of the translational start site which were hypothesized to result in the two transcripts detected (Xu et al., 2002). In addition, human *BHLHB5* contains a trinucleotide repeat sequence encoding nine serines that is not present in the mouse gene. Although no connection to a specific human disorder has yet been established, several human diseases, notably Huntington disease and spinocerebellar ataxia, have been associated with trinucleotide repeat mutations (Zoghbi, 1995; Bauer and Nukina, 2009).

Duane syndrome is a congenital eye movement disorder in which inward movement of the eye causes retraction and lateral movement of the eye is restricted. This is believed to be caused by misinnervation of the opposing medial and lateral recti by the oculomotor nerve and lack of abducens motor neurons and cranial nerve on the affected side (Tischfield et al., 2006). Mutations responsible for Duane syndrome have been narrowed to a 40kb region of the human chromosome 8q13 suggesting *BHLHB5* as a potential candidate causative gene (Calabrese et al., 2000). The *BHLHB5* locus has also been associated with hereditary spastic paraplegia (Hentati et al., 1994), a disorder characterized by progressive, generally severe, spasticity of the lower extremities (Zoghbi, 1995; Reid, 1999; Casari and Rugarli, 2001). In both Duane syndrome and hereditary spastic paraplegia, other potential candidate genes exist in the targeted area and the ultimate role of *BHLHB5* in specific disease states has yet to be determined.

Summary

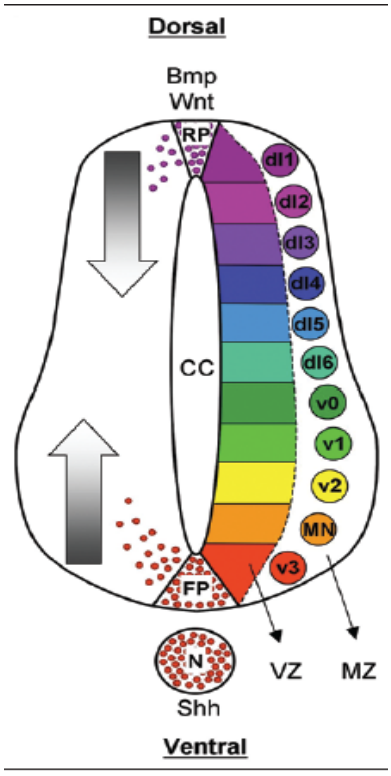
The bHLH family of transcription factors figures prominently in neural development. In spinal cord, bHLH transcription factors define progenitor domains, promote and inhibit differentiation, and participate in generation of particular neuronal subtypes. This diversity of function is carried out through diverse mechanisms such as formation of activating or inhibitory complexes and interaction with signaling pathways such as Notch. *Bhlhb5* is a novel *Olig2*-regulated bHLH gene identified as a candidate regulator of interneuron development in the embryonic spinal cord. Interneuron circuits within the spinal cord are essential for control and modulation of motor behavior but mechanisms regarding their neurogenesis are poorly understood. Investigation of the role of *Bhlhb5* in orchestrating the spatial and temporal progression of interneuron formation could greatly enhance understanding of the normal developmental mechanisms that give rise to the variety of neuronal subtypes required for the formation of functional motor circuits as well as promote understanding of pathological conditions that disrupt motor function.

Figure 1.1. Spinal cord progenitor domains

(A) Schema of a section of the spinal cord. Proliferative neuroprogenitors are located medially adjacent to the central canal (CC) in the ventricular zone (VZ). Postmitotic differentiated neurons are located laterally in the mantle zone (MZ). Distinct neuronal subtypes are generated from different domains of progenitors arrayed along the DV axis.

The spatially pattern of transcription factor expression in progenitors depends on the action of counteracting gradients of Bmp, Wnt and Shh. N, notochord; RP, roof plate; FP, floor plate. (B) Schematic of the genes that are expressed in the ventricular zone and by the differentiating and differentiated neurons that emerge from each progenitor domain of the neural tube. Adapted from Caspary & Anderson, 2003; Liu, et al., 2007; and Ulloa & Briscoe, 2007

A


Dorsal
Bmp
Wnt
RP
dI1
dI2
dI3
dI4
dI5
dI6
v0
v1
v2
MN
v3
CC
FP
N
VZ
MZ
Shh
Ventral

B

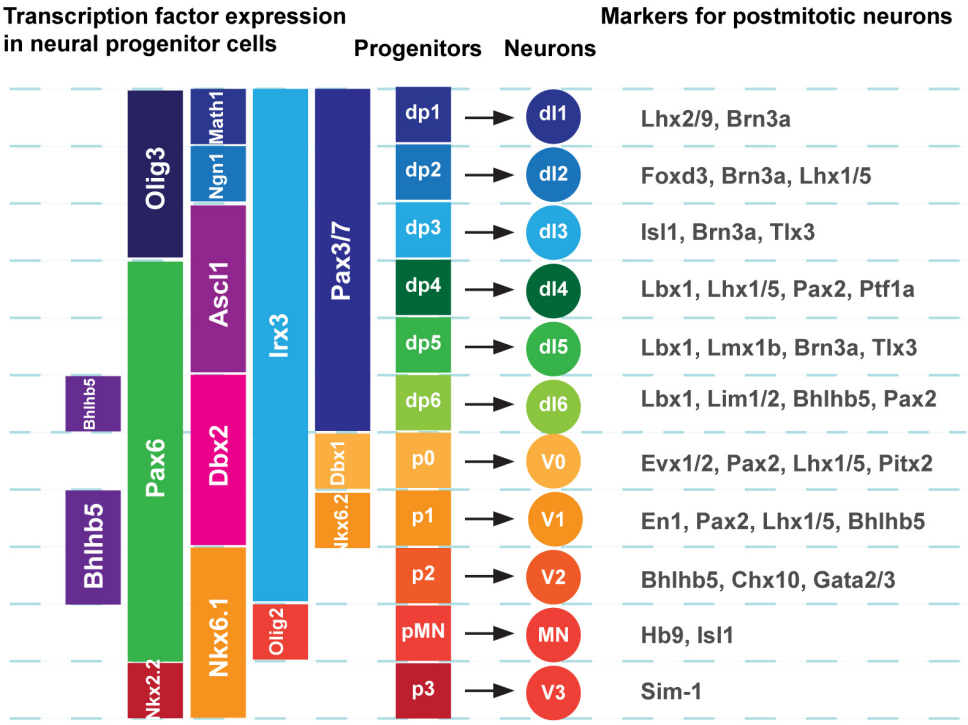

Transcription factor expression in neural progenitor cells
Progenitors
Neurons
Markers for postmitotic neurons
Olig3
Math1
Ngn1
Ascl1
Irx3
Pax3/7
Bhlhb5
Pax6
Dbx2
Nkx6.1
Olig2
Dbx1
Nkx6.2
Nkx2.2
dp1
dp2
dp3
dp4
dp5
dp6
p0
p1
p2
pMN
p3
dI1
dI2
dI3
dI4
dI5
dI6
V0
V1
V2
MN
V3
Lhx2/9, Brn3a
Foxd3, Brn3a, Lhx1/5
Isl1, Brn3a, Tlx3
Lbx1, Lhx1/5, Pax2, Ptf1a
Lbx1, Lmx1b, Brn3a, Tlx3
Lbx1, Lim1/2, Bhlhb5, Pax2
Evx1/2, Pax2, Lhx1/5, Pitx2
En1, Pax2, Lhx1/5, Bhlhb5
Bhlhb5, Chx10, Gata2/3
Hb9, Isl1
Sim-1

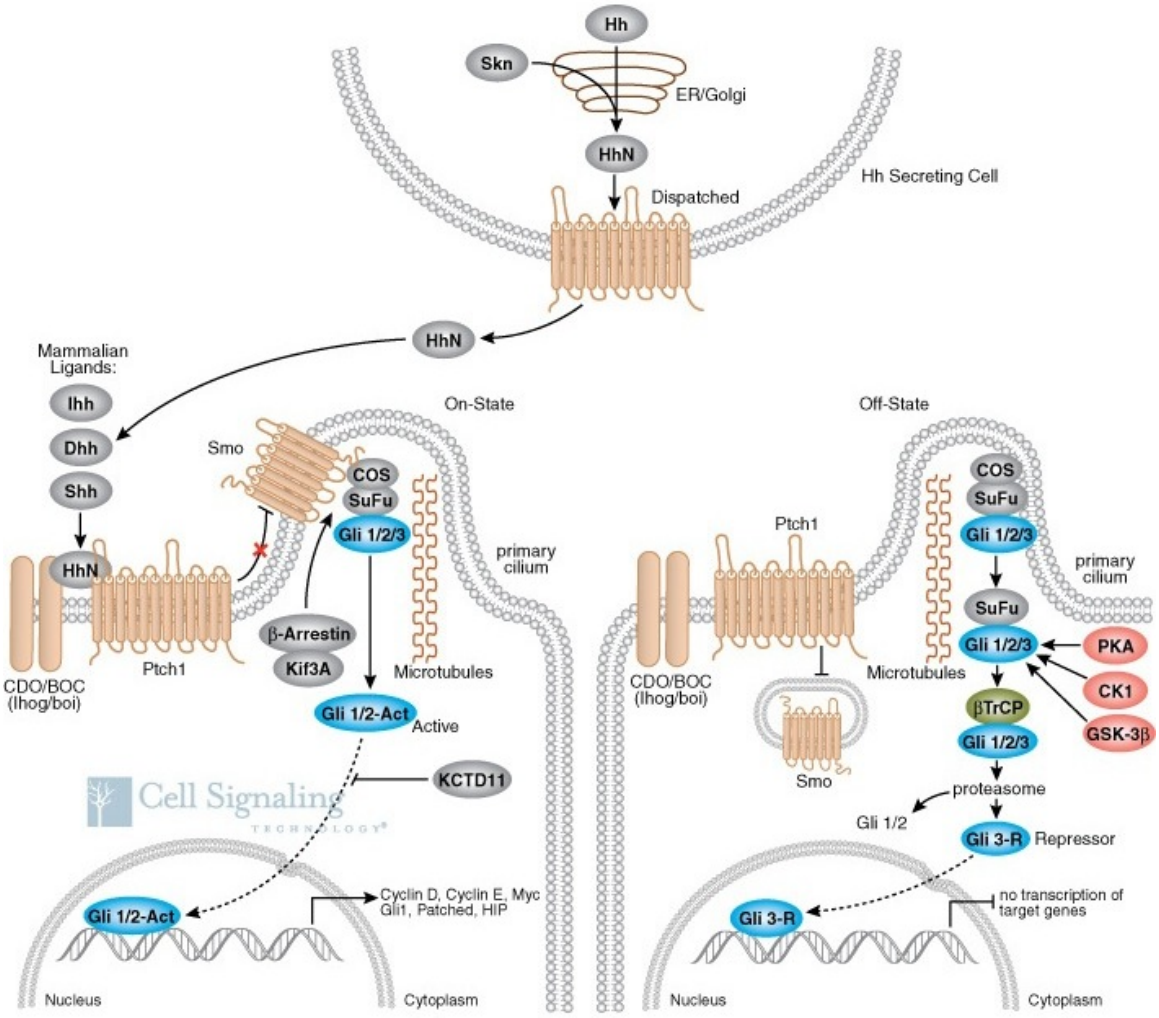
Figure 1.2. The vertebrate hedgehog signaling pathway

In the absence of hedgehog ligand in the receiving cell (Off-state), the receptor for hedgehog-family ligands, patched (Ptc1), is normally bound to and represses the activity of another transmembrane protein called smoothened (Smo).

In the Off-state, the transcription factor Gli is sequestered in the primary cilium. Phosphorylation of Gli activators (Gli1 and Gli2 in mammals) results in their degradation and Gli repressor (Gli3) actively represses hedgehog target genes.

In the On-state, hedgehog binding to patched leads to activation and translocation of smoothened to the primary cilium. It releases Gli to translocate to the nucleus and activate hedgehog target genes, including patched. The conserved action of hedgehog ligands is to switch the Gli-factors from being transcriptional repressors to activators.

Adapted from: Cell Signaling Technologies (2008). *Hedgehog signaling in vertebrates.* Retrieved from http://www.cellsignal.com/reference/pathway/Hedgehog.html.


Hh
Skn
ER/Golgi
HhN
Dispatched
Hh Secreting Cell
HhN
Mammalian Ligands:
Ihh
Dhh
Shh
On-State
Smo
COS
SuFu
Gli 1/2/3
primary cilium
HhN
Ptch1
β-Arrestin
Kif3A
Microtubules
CDO/BOC (Ihog/boi)
Gli 1/2-Act
Active
KCTD11
Cell Signaling
TECHNOLOGY
Gli 1/2-Act
Cyclin D, Cyclin E, Myc Gli1, Patched, HIP
Nucleus
Cytoplasm
Off-State
COS
SuFu
Gli 1/2/3
Ptch1
primary cilium
SuFu
Gli 1/2/3
PKA
CK1
GSK-3β
βTrCP
Gli 1/2/3
CDO/BOC (Ihog/boi)
Microtubules
Smo
proteasome
Gli 1/2
Gli 3-R
Repressor
Gli 3-R
no transcription of target genes
Nucleus
Cytoplasm

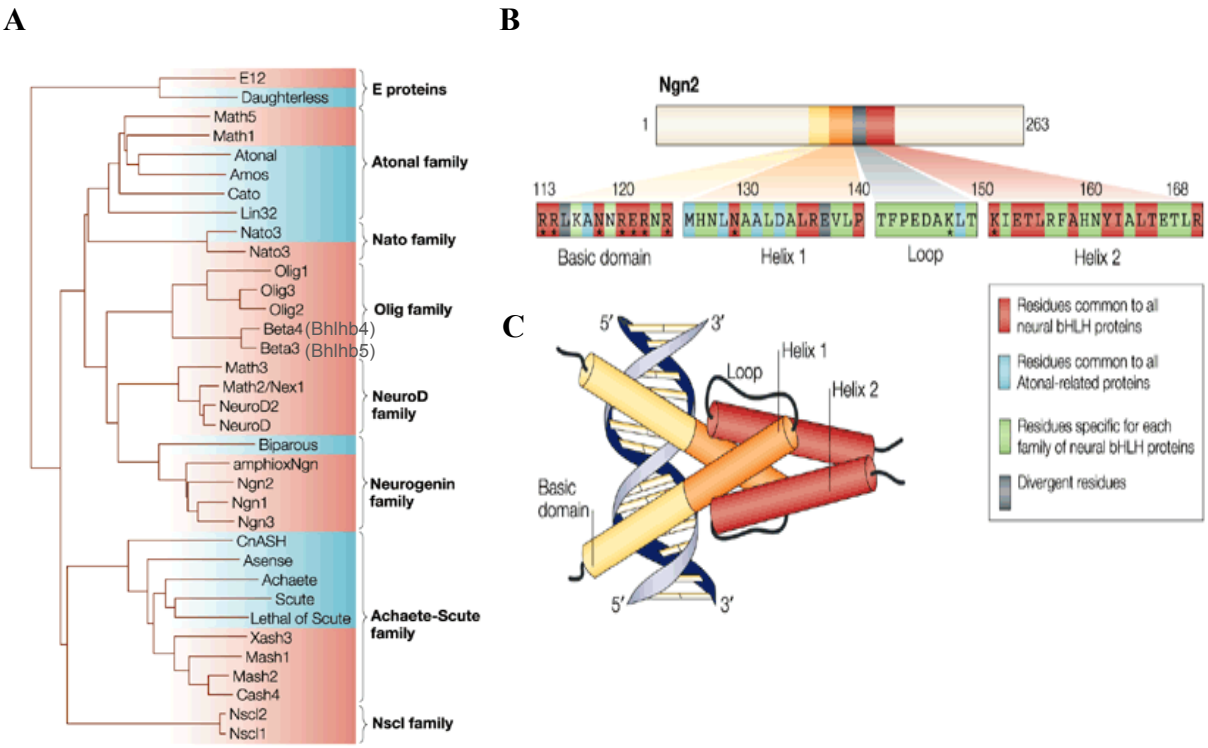


Figure 1.3. Structure and properties of neural bHLH proteins

(A) bHLH proteins grouped in distinct families on the basis of closer sequence similarities in the bHLH domain. Blue - invertebrate neural bHLH proteins. Red - vertebrate neural bHLH proteins. (B) Sequence of the bHLH domain of the mouse proneural protein neurogenin 2 (Ngn2). Color code indicates the degree of amino-acid conservation between neural bHLH proteins at each position. Asterisks mark residues that make direct contact with DNA, based on crystal structure of other bHLH proteins. (C) Schematic representation of the structure of a bHLH dimer complexed to DNA. The basic region fits in the main groove of the DNA and many residues in this region make direct contact with the E-box sequence. The two α-helices of both partners together form a four-helix bundle. Adapted from Bertrand et al., 2002.

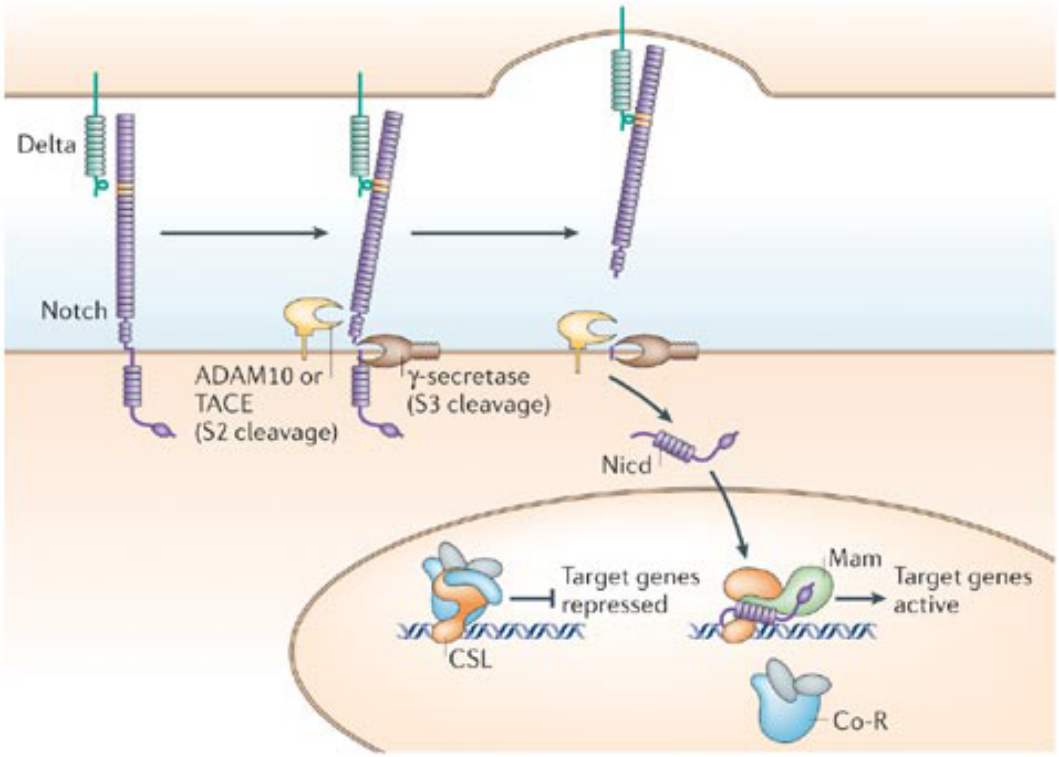


Figure 1.4. The Notch signaling pathway

Binding of the ligand on one cell to the Notch receptor on another cell results in proteolytic cleavage that releases the Notch intracellular domain. It enters the nucleus and interacts with cofactors, recruiting co-activators and releasing co-repressors to drive activation of target genes. Adapted from Bray, 2006.

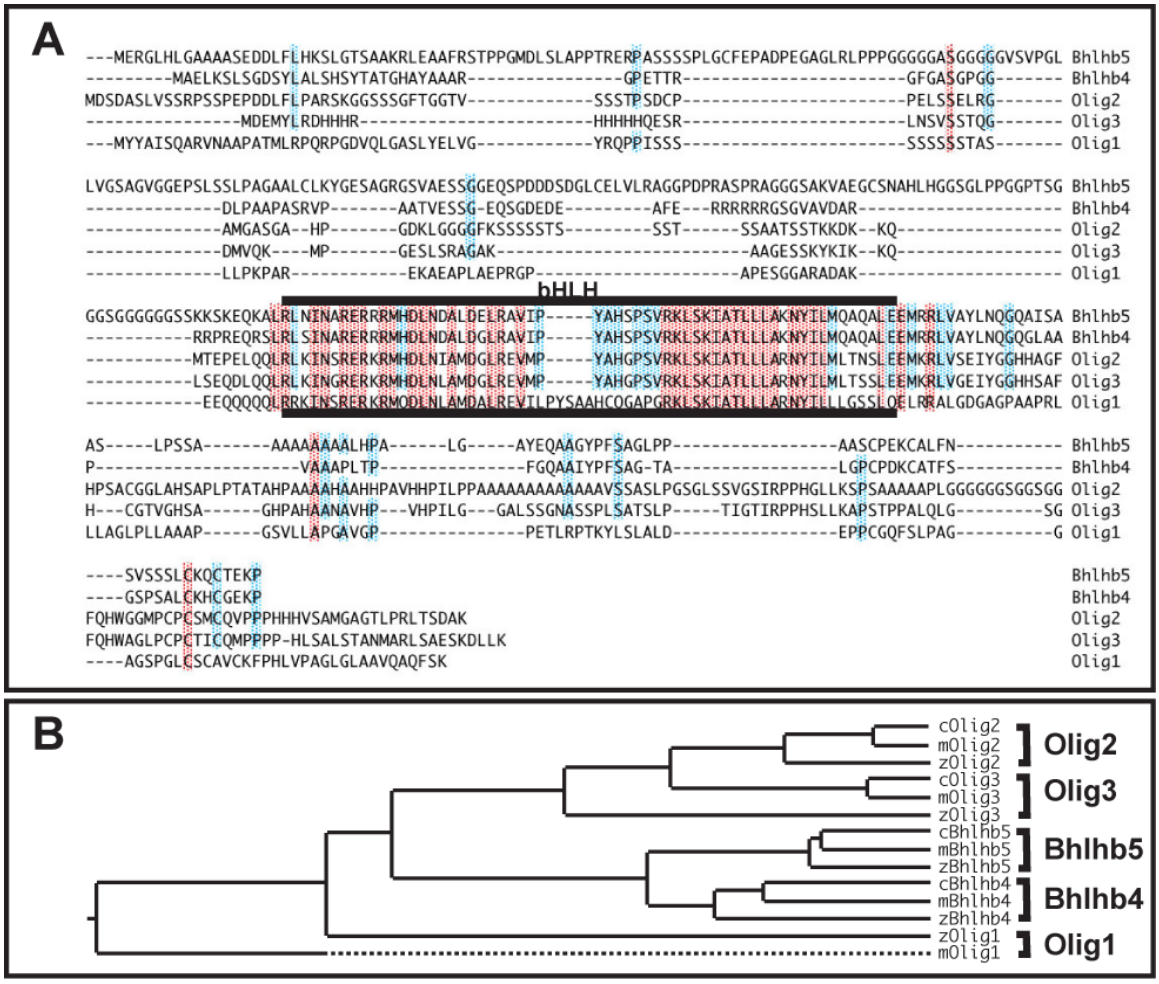


Figure 1.5. Protein alignment of the Olig family of the bHLH proteins

(A) Alignment of mouse Olig family protein using the ClustalW algorithm. Residues common to all family members are shaded in red, and those found in a majority of proteins are shaded in blue. The location of the bHLH domain is indicated by brackets. (B) Phylogentic relationship between Olig family members in chick (c), mouse (m), and zebrafish (z).

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Chapter 2

Regulation of Spinal Interneuron Development by the Olig-Related Protein Bhlhb5 and Notch Signaling[1]

Abstract

The neural circuits that control motor activities depend on the spatially and temporally ordered generation of distinct classes of spinal interneurons. Despite the importance of these interneurons, the mechanisms underlying their genesis are poorly understood. Here, we demonstrate that the Olig-related transcription factor Bhlhb5 plays two central roles in this process. Bhlhb5 repressor activity first acts downstream of retinoid signaling and homeodomain proteins to promote the formation of dI6, V1, and V2 interneuron progenitors and their differentiated progeny. In addition, Bhlhb5 is required to organize the spatially-restricted expression of the Notch ligands Jagged1, Dll1, and Dll4 that both elicit the formation of the interneuron populations that arise adjacent to Bhlhb5^{+} cells and influence the global pattern of neuronal differentiation. Through these actions, Bhlhb5 helps to transform the spatial information established by morphogen signaling into local cell-cell interactions associated with Notch signaling that control the progression of neurogenesis and extend neuronal diversity within the developing spinal cord.

Introduction

The control of vertebrate motor behaviors depends on spinal interneuron circuits that relay sensory information from the periphery and modulate motor neuron (MN) activities. This network is comprised of a diverse array of neurons defined by their expression of certain transcription factors, characteristic settling positions, projections towards different inter- and intrasegmental synaptic targets, neurotransmitter content, and effects on motor outputs (Goulding, 2009; Kiehn, 2006). To achieve the appropriate balance of excitatory and inhibitory inputs, each of these interneuron classes is generated on a precise developmental schedule and in specific numbers. While progress has been made in identifying the determinants for individual interneuron subtypes (Goulding and Pfaff, 2005; Stepien and Arber, 2008), the developmental mechanisms that orchestrate the spatial and temporal progression of their formation within the spinal cord remain poorly defined.

Much of our knowledge of spinal interneuron development has emerged from studies of the development of the ventral spinal cord. During neurulation, the combined actions of the morphogens Sonic hedgehog (Shh) and retinoic acid (RA) induce the spatially restricted expression of a series of class I (induced by RA) and class II (induced by Shh) homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors that together subdivide the neuroepithelium into 5 discrete progenitor (p) domains along the dorsoventral axis: p0, p1, p2, pMN, and p3 (Briscoe and Ericson, 2001). These progenitor groups are defined molecularly by their complement of HD and bHLH proteins and cellularly by the classes of neurons that they produce: V0-V3 interneurons and MNs (Briscoe and Ericson, 2001).

In the case of MN formation, the patterning actions of Shh and RA culminate in the expression of the bHLH transcription factor Olig2, which is uniquely expressed by pMN cells and essential for MN generation (Briscoe and Novitch, 2008; Rowitch et al., 2002). In contrast, ventral interneurons arise from multiple progenitor populations in the intermediate spinal cord that express varying combinations of the retinoid-responsive class I HD proteins Pax6, Dbx1, and Dbx2 (Ericson et al., 1997; Pierani et al., 1999; Pierani et al., 2001), and it is not clear how the combined activities of these HD proteins are integrated to elicit specific interneuron fates. Do these progenitor classes similarly depend upon equivalent Olig2-like regulators to consolidate their interneuron identities?

Using microarray screening methods to identify genes that are deregulated in *Olig2*-deficient spinal cords (Rousso et al., 2008 and data not shown), we found that the expression of the Olig-related gene *Bhlhb5* was highly increased in these mutants (see Fig. 2.9 Supplementary Figure S1). Moreover, Bhlhb5 is normally present in the interneuron subtypes that are expanded in *Olig2* mutants (see Fig. 2.9 Supplementary Figure S1 and Brunelli et al., 2003; Liu et al., 2007; Rowitch et al., 2002). Taken together, these results suggest that Bhlhb5 might play an important role in ventral interneuron development. Bhlhb5 has previously been shown to act as an essential regulator of neuronal identity in the retina and neocortex, and is required for the formation of inhibitory interneurons in the dorsal spinal cord that mediate itch (Feng et al., 2006; Joshi et al., 2008; Liu et al., 2007; Ross et al., 2010). However, its contribution to ventral spinal interneuron development has not previously been determined.

Once formed, neural progenitor domains in the spinal cord appear to have the capacity to produce multiple classes of neurons as well as glial cells later in development

(Del Barrio et al., 2007; Hochstim et al., 2008; Muroyama et al., 2005; Peng et al., 2007; Rousso et al., 2008). This additional level of neuronal diversification has been attributed to two distinct mechanisms: different MN subtypes are generated by the actions of diffusible morphogens on the postmitotic cells, which induce the patterned expression of LIM-HD, Hox, and Forkhead domain transcription factors that together define individual MN fates (Agalliu et al., 2009; Dasen et al., 2008; Dasen et al., 2003; Rousso et al., 2008; Sockanathan and Jessell, 1998). In contrast, V2 spinal interneurons segregate into two distinct classes, excitatory V2a neurons and inhibitory V2b neurons, through local cell-cell signaling mediated by Dll4 and Notch receptors (Batista et al., 2008; Del Barrio et al., 2007; Kimura et al., 2008; Peng et al., 2007). While Notch receptors are broadly expressed throughout the spinal cord, Notch ligands and Fringe proteins, which modulate Notch receptor functions, are expressed in a domain-restricted manner (Lindsell et al., 1996; Marklund et al., 2010; Myat et al., 1996; Rocha et al., 2009; Yeo and Chitnis, 2007). These observations raise the questions of how are these precise patterns established, and to what extent does the localized expression of Notch ligands contribute to interneuron fate determination and the regional control of neurogenesis within the spinal cord?

In this study, we demonstrate that Bhlhb5 provides a critical link between the early patterning actions of retinoid signaling and its effector Pax6 that both specifies the neural progenitors that give rise to dI6, V1, and V2a spinal interneurons and spatially organizes the expression of Notch ligands and Fringe proteins. When misexpressed with proneural bHLH proteins, Bhlhb5 repressor function directs the ectopic formation of these specific classes of interneurons, while the acute loss of Bhlhb5 disrupts their

development. Modulation of Bhlhb5 function further alters the pattern of Notch ligand and Fringe expression leading to broad changes in Notch pathway activity and neurogenesis in the intermediate regions of the spinal cord. Together, these findings provide evidence that Bhlhb5 contributes to the identity and timing of spinal interneuron differentiation through both direct and indirect mechanisms linked to the Notch signaling pathway.

Materials and Methods

Animal preparation and tissue analysis

Fertilized chicken eggs (Michigan State University Poultry Farm) were incubated, staged, and electroporated at embryonic day 2 (E2; HH stages 11-13) or E3 (HH stages 17-18) and analyzed at E5 (HH stage 27) as previously described (Novitch et al., 2001; Rousso et al., 2008) unless otherwise indicated. *Olig2*$^{GFP/+}$, *Pax6*$^{Sey/+}$, and *Dbx1*$^{LacZ/+}$ mice were maintained and mated for embryo collection as previously described (Ericson et al., 1997; Mukouyama et al., 2006; Pierani et al., 2001). All embryos were fixed in 4% paraformaldehyde, cryosectioned, and processed for antibody staining or in situ hybridization histochemistry as previously described (Novitch et al., 2001; Rousso et al., 2008). Primary antibodies used are listed in Table S1. Many of the monoclonal antibodies used were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa.

Expression constructs

A Bhlhb5 expression vector was generated by PCR subcloning the mouse *Bhlhb5* gene into the pCIG expression vector (Megason and McMahon, 2002). Constitutive activator and repressor forms of Bhlhb5 were generated by fusing the Bhlhb5 bHLH domain (amino acids 208-288) in frame to either the Herpes Virus VP16 transactivation domain or the Drosophila Engrailed repressor domain (Novitch et al., 2001), and inserting these products into a Gateway cloning compatible version of pCIG (pCIG-gw). The Bhlhb5 Δb construct was achieved by deletion of amino acids 221-228 through overlap extension PCR mutagenesis. The additional expression constructs used were either previously described (Novitch et al., 2001; Novitch et al., 2003), or generated by PCR cloning cDNAs into pCIG, pCIG-gw, or RCAS expression vectors.

shRNA-mediated gene knockdown

Three shRNAs were created against the endogenous chick *Bhlhb5* sequence (sh1: 5'-tggagcattgcttacggaaga-3'; sh2: 5'-ggaaatctcttgaaggtgaat-3'; sh3: 5'-tttaagcgactcgcggaaaca-3') using the selection criteria outlined in Das et al., 2006. Each was synthesized via PCR and inserted into the pRFP-RNAi vector (Das et al., 2006), or a modified form of this vector in which the RNAi cassette had been moved into the pCIG backbone. Scrambled shRNA controls were generated by randomizing the nucleotide sequence of each shRNA (sh1 scrambled: 5'-tgctggaaaagttcgagtacg-3'; sh2 scrambled: 5'-agtggcgaagtaattacgatg-3'; sh3 scrambled: 5'-cttgacagatatgagaagccc-3'). An additional non-specific control shRNA was generated based on the sequence 5'-

cagtcgcgtttgcgactgg-3' that lacks similarity to known mammalian and chick genes (Yuan et al., 2007 and data not shown).

In situ hybridization probes

Digoxigenin-labeled in situ hybridization probes were generated from chick embryonic spinal cord cDNA using PCR and primers directed against the 3'UTR of chick *Scl* (forward primer: 5'-gaggaattcggacgctcggtgtttaggta-3'; reverse primer: 5'-gagattaaccctcactaaagggactttgctgagggcattttc-3'), chick *Dll4* (forward primer: 5'-cttctgggccatgtgagaat-3'; reverse primer: 5'- gagtatttaggtgacactataggttccatcctccacctgaga-3'), chick *Lfng* (forward primer: 5'-ccttggtcagggtcacagtt-3'; reverse primer: 5'-gagaattaaccctcactaaagggagaggggcacctgtgttttta-3'), and chick *Hes5* isoform 2 (*Hes5-2)* (forward primer: 5'- ttccaaaggaaaaaccaacg-3'; reverse primer: 5'-gagattaaccctcactaaagggaacgtctgtagcgacccttg-3'). Underlined regions indicate a SP6 or T3 RNA polymerase site embedded in the reverse primers. The mouse *Bhlhb5* in situ probe was generated from EST clone BM950131. mRNA signals were quantified using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Results

Bhlhb5 is expressed in distinct progenitor domains in the developing spinal cord and retained by subsets of differentiating interneurons

To assess whether Bhlhb5 might play a role in ventral interneuron fate determination, we first generated antibodies to the chick and mouse proteins to track its expression at key stages of spinal cord development. In the chick, Bhlhb5 was first

detected shortly after neural tube closure at embryonic day 2 (E2; Hamburger-Hamilton (HH) stage 10) in a group of cells dorsal to Olig2^{+} pMN domain (Fig. 2.1A). Bhlhb5 expression coalesced into two distinct progenitor stripes by E3 (HH stages 17-19; Fig. 2.1B), and a third stripe containing scattered Bhlhb5^{+} cells became detectable immediately dorsal to the pMN by between E3-E5 (HH stages 23-27; Figs. 2.1C,D). There appeared to be significant differences in the intensity of Bhlhb5 staining within these progenitors, as Bhlhb5 was most highly expressed in the dorsal-most stripe and weakest in the middle stripe (Figs. 2.1C,D). Bhlhb5 and Olig2 remained exclusive of one another throughout early embryogenesis (Figs. 2.1D-F and data not shown). At gliogenic stages such as E11 (HH stage 37) and later, nearly all Bhlhb5^{+} cells expressed the neuronal marker NeuN, though a few Bhlhb5^{+} cells lacked NeuN staining and instead expressed the astrocyte progenitor marker NF1A (data not shown; Deneen et al., 2006). Thus, Bhlhb5 appears to mark several populations of interneuron progenitors and differentiated neurons in the intermediate spinal cord, as well as a small number of astrocyte progenitors, reminiscent of the sequential expression of Olig2 in MN and oligodendrocyte progenitors.

Previous reports have suggested that Bhlhb5 expression is confined to postmitotic cells in both the retina and spinal cord (Feng et al., 2006; Liu et al., 2007; Ross et al., 2010). However, in the E4 chick Bhlhb5 overlapped with the neural progenitor marker Sox2 (Figs. 2.1G,H; Bylund et al., 2003; Graham et al., 2003), and these cells were readily labeled with a 30-minute pulse of 5-bromo-2-deoxyuridine (BrdU) administered *in ovo* (Fig. 2.1J). Nevertheless, Bhlhb5 was also present in actively differentiating neurons marked by the proneural bHLH proteins Neurogenin2 (Ngn2) and NeuroM, and

postmitotic cells expressing the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Figs. 2.1G,I,K,L). Similar results were observed in sections of E9.5-E10.5 mouse spinal cords, where low levels of Bhlhb5 protein were detected in Sox2 and Ki-67^{+} progenitors and higher levels seen in differentiated cells (see Fig. 2.10 Supplementary Figure S2A-E), indicating that in both species Bhlhb5 is initially expressed by dividing progenitors and then maintained by specific neuronal populations.

We next mapped the expression of Bhlhb5 with respect to HD transcription factors that demarcate discrete interneuron progenitor domains in the chick spinal cord. Bhlhb5 was closely associated with the expression of Pax6 in several interneuron progenitor domains from pdI6 dorsally to p2 ventrally (Figs. 2.2A,N; Ericson et al., 1997). The Pax6 region is subdivided by Dbx2, which spans the pdI6 through p1 domains, and Dbx1, which is confined to the p0 domain (Pierani et al., 1999; Pierani et al., 2001). Bhlhb5 was present in the dorsal- and ventral-most $Dbx2^{+}$ progenitors, but was not coexpressed with Dbx1 (Figs. 2.2B,C,N). The ventral extent of Bhlhb5 expression coincided with $Nkx6.1^{+}$ p2 and $Nkx2.2^{+}$ p3 interneuron progenitors, but did not extend into the $Olig2^{+}$ pMN domain (Fig. 2.1C; Figs. 2.2D,E,N).

By E5 (HH stage 27) and later developmental stages, Bhlhb5 was prominently associated with several populations of interneurons that expressed the LIM-HD proteins Lhx1 and Lhx5 (Fig. 2.2F). Using a panel of markers for the known interneuron subpopulations at this stage, we determined that Bhlhb5 was present in the ventral-most group of $Lbx1^{+}$ cells, presumed to be dI6 interneurons, yet absent from more dorsal $Lbx1^{+}$ cells such as $Lmx1b^{+}$ dI5 interneurons (Figs. 2.2G,H; Gross et al., 2002; Muller et al., 2002). Bhlhb5 was missing from $Evx1^{+}$ V0 interneurons (Fig. 2.2I), but was

expressed by other ventral interneuron populations including a subset of $En1^+$ V1 interneurons and $Chx10^+$ V2a interneurons (Figs. 2.2J,K; Briscoe et al., 2000; Del Barrio et al., 2007; Peng et al., 2007). However, Bhlhb5 was notably absent from most $Gata3^+$ and Scl^+ V2b interneurons (Figs. 2.2L,M; see also Fig. 2.11 Supplementary Figure S3). Bhlhb5 was expressed earlier than the previously characterized markers of V2 neurons such as FoxN4, Gata2 and Lhx3 (see Fig. 2.11 Supplementary Figure S3 and Del Barrio et al., 2007; Li et al., 2005; Peng et al., 2007), suggesting that its expression precedes the division of these cells into the V2a and V2b subclasses. A few cells that contained low levels of both Bhlhb5 and Gata3 were nevertheless detectable in this analysis (see Fig. 2.11 Supplementary Figure S3L), most likely reflecting cells in transition towards the V2b fate. Bhlhb5 expression was lastly seen in a subset of V3 interneurons, but absent from differentiated MNs (Fig. 2.2E and data not shown). With the exception of the V3 interneuron expression, Bhlhb5 was similarly expressed in the mouse spinal cord (see Fig. 2.10 Supplementary Figure S2F-L), indicating an evolutionarily conserved association of Bhlhb5 with dI6, V1, V2a progenitors and their differentiated progeny (Fig. 2.2N; see also Fig. 2.10 Supplementary Figure S2M).

Bhlhb5 expression depends on retinoid signaling and Pax6 activity, and is spatially confined by both Olig2 and Dbx1

We next sought to determine how the discontinuous expression of Bhlhb5 in interneuron progenitors is established. Retinoid signaling and Pax6 transcriptional activity provide the major stimulus for interneuron development in the intermediate region of the spinal cord (Ericson et al., 1997; Pierani et al., 1999). To examine the

relationship between these factors and Bhlhb5, we used *in ovo* electroporation approaches in chick to misexpress a dominant-negative form of the RA receptor α (dnRAR) that blocks RA signaling in vivo (Novitch et al., 2003). This construct potently suppressed Pax6 and Bhlhb5 (Figs. 2.3A,B), indicating that both factors are retinoid-dependent. Coelectroporation of dnRAR along with Pax6 expression constructs restored Bhlhb5 levels (Figs. 2.3C,D), indicating that Pax6 acts downstream of retinoid signaling to promote Bhlhb5 expression. Consistent with this conclusion, Bhlhb5 levels were dramatically reduced in *Pax6*$^{Sey/Sey}$ mutant mice compared to littermate controls, and closely mirrored the interneuron deficit seen in these animals (Figs. 2.3E,F, and data not shown; Ericson et al., 1997).

The progenitor domains in the spinal cord are established by cross-repressive interactions of class I and class II transcription factors that act downstream of RA and Shh signaling (Briscoe and Novitch, 2008). Olig2, for example, opposes both Nkx2.2 and Irx3 to define the borders of the p3, pMN, and p2 progenitor domains (Mizuguchi et al., 2001; Novitch et al., 2001). We therefore sought to determine whether similar interactions occur between Bhlhb5 and the proteins that define its neighboring progenitor domains. Olig2 misexpression in the chick strongly repressed Bhlhb5, while Bhlhb5 expanded ventrally in the spinal cord of *Olig2* mutant mice (Figs. 2.3G-J; see also Fig. 2.9 Supplementary Figure S1A-J). When Bhlhb5 was similarly misexpressed, Olig2 was decreased (Figs. 2.3M,Q), suggesting that cross-repressive interactions between Olig2 and Bhlhb5 help maintain the boundary between the p2 and pMN domains.

To account for the exclusion of Bhlhb5 from p0 progenitors and V0 interneurons, we next examined *Dbx1*^{lacZ} knockout mice which lack these cells (Pierani et al., 2001).

In *Dbx1*$^{lacZ/+}$ heterozygous controls, Bhlhb5 expression was excluded from most β-Galactosidase (β-Gal)$^{+}$ p0 progenitors and their derivatives (Fig. 2.3K). However, in *Dbx1*$^{lacZ/lacZ}$ homozygous mutants, Bhlhb5 expression expanded into many of the β-Gal^{+} cells (Fig. 2.3L). The interactions between Dbx1 and Bhlhb5 were reciprocal, since Bhlhb5 misexpression potently suppressed Dbx1 and to a much lesser extent Dbx2 though it did not alter other class I HD proteins such as Pax6, Pax7, or Irx3 revealing specificity to its actions (Figs. 2.3M-P and data not shown). Thus, Bhlhb5 appears to be promoted by both retinoid signaling and Pax6 function, and restricted from specific progenitor domains by repressive interactions with Dbx1/2 and Olig2 (Fig. 2.3R).

Misexpression of Bhlhb5 with Neurogenins leads to ectopic generation of dI6, V1, and V2a interneurons and suppresses the development of other neuronal classes

The selective expression of Bhlhb5 in the progenitors that give rise to dI6, V1, and V2a interneurons and the correlation of changes of Bhlhb5 expression with altered interneuron development in *Olig2* mutants raised the possibility that Bhlhb5 might act as a determinant of these fates. Supporting this idea, misexpression of Bhlhb5 along with an IRES-nuclear EGFP (nEGFP) reporter throughout the chick spinal cord led to the formation of a small number of ectopic Chx10^{+} V2a interneurons in the dorsal spinal cord and ectopic Lbx1^{+} cells in the ventral spinal cord, presumed to be dI6 interneurons due to their position and lack of Lmx1b expression (Figs. 2.4A-C,T,U,W; see also Fig. 2.12 Supplementary Figure S4A-D). In contrast, other cell populations such as Evx1^{+} V0, En1^{+} V1, and Gata3^{+}/Scl^{+} V2b interneurons were markedly reduced (Figs. 2.4D,E,T,V; see also Fig. 2.12 Supplementary Figure S4E-G and Fig. 2.14 Supplementary Figure S6).

The net loss of interneuron subtypes generally exceeded the gains seen in the V2a and dI6 populations, raising the possibility that Bhlhb5 misexpression might be either toxic to cells or capable of impairing neuronal differentiation. Bhlhb5 misexpression did not increase apoptotic death measured by antibody staining for the activated form of caspase-3 (data not shown), but it did reduce the expression of Ngn2 and the appearance of postmitotic neurons by ~10-20% (Fig. 7X), indicating that Bhlhb5 can suppress neurogenesis when overexpressed.

Previous work in cultured cells has indicated that high levels of Bhlhb5 expression can mimic the antineural activity of the Id protein family in blocking interactions between proneural bHLH transcription factors and their E protein DNA binding partners (Peyton et al., 1996; Ruzinova and Benezra, 2003; Xu et al., 2002). However, an Id-like function for endogenous Bhlhb5 seems unlikely as it is normally coexpressed with Ngn2 and its downstream target NeuroM as interneurons begin to differentiate and then expression is retained in postmitotic neurons (Figs. 2.1I,K,L). Nevertheless, we reasoned that the fate-specifying activity of exogenously expressed Bhlhb5 in interneuron fate determination might be better assessed if the balance between Bhlhb5 and proneural proteins was restored in these gain-of-function assays by concomitantly misexpressing Bhlhb5 with Ngn1 or Ngn2 (collectively referred to hereafter as Ngn). The combined expression of Bhlhb5 with either Ngn resulted in a dramatic production of V2a interneurons throughout the spinal cord (Figs. 2.4K-M,W; see also Fig. 2.12 Supplementary Figure S4CC), and enhanced the appearance of ectopic dI6-like and V1 interneurons in the ventral spinal cord, though the numbers of these cells in their endogenous positions were typically reduced (Figs. 2.4Q,S,U,V; see also Fig.

2.12 Supplementary Figure S4BB,EE). All of the ectopic cells expressed the GFP transfection marker, indicating that these effects are due to the cell autonomous actions of Bhlhb5 and Ngn (data not shown). Bhlhb5 and Ngn coexpression consistently inhibited the formation of other ventral interneuron subtypes such as V0 and V2b interneurons, much like that seen with Bhlhb5 misexpression alone (Figs. 2.4D,E,N,O,R,T; see also Fig. 2.12 Supplementary Figure S4FF,GG), suggesting that the loss of the endogenous dI6, V0, V1, and V2b interneurons may result from a conversion of these cells into V2a interneurons. Supporting this conclusion, we observed that Bhlhb5 misexpression reduced the expression of the critical V2b fate determinants Scl and Foxn4 (Fig. 4E and Fig. 2.13 Supplementary Figure S5C), indicating that Bhlhb5 acts at an early stage in V2 interneuron fate selection.

Misexpression of Ngn by itself promoted neuronal differentiation as expected, but did not alter the assignment of most specific interneuron fates with the exception of $Lmx1b^{+}$ $Lbx1^{+}$ dI5 interneurons, which were suppressed whether or not Bhlhb5 was present, and $Gata3^{+}$ V2b interneurons, which were slightly increased (Figs. 2.4F-J,T-W; see also Fig. 2.12 Supplementary Figure S4H-N and Fig. 2.13 Supplementary Figure S5D). The observed effects of Bhlhb5 and Ngn misexpression on interneuron fate assignment were not recapitulated by the coexpression of Id1 and Ngn (see Fig. 2.12 Supplementary Figure S4O-U,HH-MM), confirming that the actions of Bhlhb5 are distinct from that exhibited by Id proteins. Collectively, these results indicate that the combined transcriptional activities of Bhlhb5 and Ngn are sufficient to direct the differentiation of V2a interneurons, and to a lesser extent dI6 and V1 interneurons, and suppress the formation of other neuronal classes.

Bhlhb5 promotes dI6 and V2a interneuron formation through its transcriptional repressor activity

Members of the Olig gene family direct specific neuronal fates through their function as transcriptional repressors (Muller et al., 2005; Novitch et al., 2001), raising the question of whether Bhlhb5 acts in a similar manner to promote dI6, V1, and V2a interneuron formation. We therefore created dominant repressor and activator forms of Bhlhb5 by fusing its bHLH DNA binding region to either the transcriptional repressor domain from the Drosophila Engrailed protein (EnR) or the Herpes Virus VP16 transcriptional activation domain (Fig. 2.5A; Novitch et al., 2001). To further confirm that Bhlhb5 requires DNA binding activity, we created a DNA binding mutant form of full-length Bhlhb5 lacking the basic region (Bhlhb5$^{\Delta b}$). When coexpressed with Ngn2, Bhlhb5-EnR produced a large number of ectopic V2a and dI6-like interneurons throughout the spinal cord, and potently suppressed the formation of V0 and V2b interneurons (Figs. 2.5B-F). Thus, Bhlhb5-EnR is capable of mimicking the actions of the full-length Bhlhb5 protein. In contrast, neither Bhlhb5-VP16 nor Bhlhb5$^{\Delta b}$ significantly affected the pattern of interneuron formation aside from minor reductions in cell numbers (Figs. 2.5G-P and data not shown). Thus, the ability of Bhlhb5 to regulate spinal interneuron development depends on its ability to bind and repress specific DNA targets.

Knockdown of Bhlhb5 expression disrupts the development of multiple interneuron classes

We next sought to determine whether endogenous Bhlhb5 function is required for interneuron generation using gene knockdown approaches in chick embryos. We used a plasmid vector to deliver three different short hairpin RNA (shRNA) constructs against the untranslated regions of the chick *Bhlhb5* gene (Fig. 2.6A) or scrambled shRNA controls. Whether expressed individually or in combination, the *Bhlhb5* shRNAs decreased the number of cells expressing Bhlhb5 by ~50%, and the protein that remained was reduced compared to the non-transfected side of the spinal cord (Figs. 2.6B,C,T; see also Fig. 2.13 Supplementary Figure S5H,I,O,P,V,W).

Despite its ability to alter the dorsoventral patterning of the spinal cord when misexpressed (Fig. 2.3), Bhlhb5 knockdown did not lead to any significant change in the initial establishment interneuron progenitor domains (Fig. 2.7W, Fig. 2.13 Supplementary Figure S5G,H, and data not shown), suggesting that its contributions to this process are redundant with those mediated by class I and II homeodomain proteins. Nevertheless, the loss of Bhlhb5 coincided with a ~50% decrease in all of the neuronal subtypes with which Bhlhb5 is normally associated, including V1, V2a, and dI6 interneurons (Figs. 2.6D,G,J,T).

Intriguingly, knockdown of Bhlhb5 also appeared to reduce the generation of V0 interneurons and V2b interneurons (Figs. 2.6F,J,T and Fig. 2.13 Supplementary Figure S5F), which do not normally express Bhlhb5 (Figs. 2.2I,L). However, not all neurons were affected by Bhlhb5 knockdown, as there was little change to the numbers of dI5 interneurons and MNs formed (Figs. 2.6E,H,T). Similar results were achieved with the

individual expression of the three *Bhlhb5* shRNA constructs but not with scrambled shRNA sequences or a control shRNA vector (Figs. 2.6K-T, Fig. 2.14 Supplementary Figure S6A-G, and data not shown), indicating that these results are specific to the loss of *Bhlhb5* and unlikely to result from off-target effects. Moreover, we did not observe any significant increase in apoptotic cell death that could account for these results (data not shown), and the suppressive effects of Bhlhb5 knockdown were rescued by the misexpression of a mouse *Bhlhb5* cDNA lacking the shRNA binding sites (see Fig. 2.14 Supplementary Figure S6CC-II). Together with our misexpression results, these data suggest that cell-autonomous functions of Bhlhb5 are required for the formation of dI6, V1, and V2a interneurons, while non-cell-autonomous functions influence the generation of interneuron populations that form adjacent to Bhlhb5^{+} cells.

Bhlhb5 regulates the pattern of Notch ligand expression, Notch pathway activity, and the onset of neuronal differentiation in the spinal cord

The broad reduction of interneuron subtypes observed in the Bhlhb5 knockdown experiments could be explained by either a general change in neuronal differentiation, much as is seen with Bhlhb5 misexpression, or a loss of instructive signals produced by Bhlhb5^{+} cells to promote the formation of specific interneuron subtypes. As the spinal cord develops, Notch ligands and Fringe proteins are expressed in domain-specific patterns that suppress Notch signaling across domain boundaries while facilitating Notch activation within each domain (Marklund et al., 2010). Moreover, within the V2 interneuron lineage, cell-cell communication mediated by Dll4 and Notch receptors further enables the emergence of V2a and V2b neurons from a common progenitor pool

(Del Barrio et al., 2007; Peng et al., 2007; Rocha et al., 2009). To explore whether the observed non-autonomous effects of Bhlhb5 misexpression and deficiency might be attributed to changes in the focal expression of Notch ligands, we mapped the expression of Bhlhb5 relative to Jagged1, Dll1, and *Dll4* and the Notch regulator L*unatic Fringe* (*Lfng*) in the unaltered chick spinal cord and found that Bhlhb5 expression strikingly overlaps Jagged1 and is reciprocal to both Dll1 and *Lfng* (Figs. 2.7A,B,D). A subset of ventral Bhlhb5^{+} cells in the p2 region, however, lacked both ligands and instead expressed *Dll4* mRNA (Fig. 2.7C and data not shown), consistent with the reported association of *Dll4* with V2a interneurons (Del Barrio et al., 2007; Peng et al., 2007). Thus, Bhlhb5 appears to be closely associated with the domain-restricted pattern of both Notch ligands and *Lfng* expression within the spinal cord (Fig. 2.7F).

To examine how Bhlhb5 contributes to this pattern, we examined embryos in which Bhlhb5 had been misexpressed. The ectopic expression of Bhlhb5 significantly reduced the expression of both Dll1 and Lfng, and simultaneously increased the expression of *Dll4* and to a lesser extent Jagged1 (Figs. 2.7G-J). These changes in Notch ligand distribution coincided with an elevation of Notch pathway activity in the intermediate spinal cord, reflected by a ~40% increase in the expression of the downstream effector *Hes5* and a corresponding reduction in the number of Ngn2^{+} progenitors and p27^{Kip1+} neurons formed (Figs. 2.7K,L,X,Z). A similar reduction of Dll1 and Lfng and increase in *Dll4* was seen with the combined expression of Bhlhb5 and Ngn2, but not with misexpression of Ngn alone (see Fig. 2.15 Supplementary Figure S7), indicating that these effects are specific to Bhlhb5 misexpression and not simply a consequence of having forced cells to differentiate.

Complementing these misexpression results, we found that endogenous Bhlhb5 function is required for the alternating pattern of Notch ligands in the spinal cord. shRNA-mediated knockdown of Bhlhb5 decreased *Dll4* and Jagged1 expression, and both Dll1 and Lfng expanded into the territories where *Dll4* and Jagged1 would normally be expressed (Figs. 2.7M-P,R,S and data not shown). Under these conditions, *Hes5* expression was reduced by ~50% while Ngn2 and NeuroM expanded, and $p27^{Kip1+}$ postmitotic cells began to abnormally appear within the ventricular zone (Figs. 2.7Q,R,T,U,V,X). These changes in neuronal differentiation also extended into the $Dbx1^{+}$ p0 progenitor domain (Figs. 2.7W,Y), suggesting that the observed alterations in Notch ligand and Lfng distribution can broadly affect Notch pathway activity and neurogenesis even in regions where Bhlhb5 is not normally expressed. Together, these results provide evidence that in addition to its cell-autonomous functions in promoting dI6, V1, and V2a interneuron formation, Bhlhb5 regulates the expression of Notch ligands and can thus influence the overall pattern of neuronal differentiation in the spinal cord. Moreover, in directing *Dll4* expression, Bhlhb5 provides an instructive stimulus for V2b neuron development, thereby extending the repertoire of interneuron subtypes generated.

Discussion

The interneuron circuits that modulate motor activities result from the spatially and temporally ordered generation of distinct classes of neurons in the ventral spinal cord. This process begins with the establishment of discrete groups of progenitors along the dorsoventral axis and subsequently requires cell-cell communication within these

progenitor domains to coordinate the timing of their differentiation and extend the variety of neuronal subtypes formed. Our results show that the bHLH transcription factor Bhlhb5 helps orchestrate this progression by participating in the initial formation of progenitor domains, directing the generation of specific classes of spinal interneurons, and organizing the expression of Notch ligands to regulate the global pattern of neurogenesis and enhance neuronal diversity (Fig. 2.8). We discuss below the contributions of Bhlhb5 to each of these processes.

Olig/Bhlhb family proteins as links between morphogen signaling and the assignment of neuronal fates

Our data together with previous studies of the related proteins Olig2 and Olig3 (Mizuguchi et al., 2001; Muller et al., 2005; Novitch et al., 2001) suggest a common function for Olig/Bhlhb transcription factors in translating early patterning information into distinct programs of neuronal differentiation throughout the spinal cord. In MN progenitors, Shh and RA signaling act through the HD proteins Pax6 and Nkx6 to induce the expression of Olig2, whose function defines the borders of this domain and promotes MN differentiation (Fig. 2.8A) (see also Mizuguchi et al., 2001; Novitch et al., 2001). Olig3 is activated similarly by Bmp and Wnt signaling in the dorsal spinal cord, where it directs the formation of dI1-dI3 interneurons and suppresses dI4-dI6 fates (Muller et al., 2005; Zechner et al., 2007). Bhlhb5 appears to play an analogous role in the intermediate spinal cord, acting as a downstream effector of RA signaling and Pax6 activity that helps to establish or maintain progenitor domain borders through its repressive interactions

with Dbx1 and Olig2, and promote dI6, V1, and V2a interneuron fates in lieu of others (Fig. 2.8A).

It is notable that Bhlhb5, Olig2, and Olig3 all carry out their patterning and fate specifying functions as transcriptional repressors (Muller et al., 2005; Novitch et al., 2001), suggesting that this is yet another defining feature of this protein family. The repressive actions of Bhlhb5 and its closest paralog Bhlhb4 have previously been attributed to their ability to block the formation of proneural bHLH-E protein DNA binding complexes, akin to the Id family of inhibitory HLH proteins (Bramblett et al., 2002; Ohkawara et al., 2004; Peyton et al., 1996; Xu et al., 2002). However, our data indicate that Bhlhb5 activity in the developing spinal cord depends on its ability to bind DNA targets and repress transcription. Consistent with this conclusion, we found that Id proteins are unable to recapitulate the function of Bhlhb5 in neuronal fate specification. Together, these results support a model in which Bhlhb5 promotes the development of specific interneuron classes by suppressing genes that are broadly antagonistic to interneuron development or associated with alternative interneuron fates.

Our loss-of-function experiments in the chick also suggest a requirement for Bhlhb5 activity within the intermediate spinal cord, as many classes of spinal interneurons were diminished when Bhlhb5 expression was reduced. Bhlhb5 knockdown effects were consistently achieved using various shRNA constructs targeting different regions of the transcript. However, the reduction of Bhlhb5 in our experiments was never complete and, perhaps due to this limitation, the formation of different classes of interneurons was greatly reduced but not completely lost. The persistence of interneuron development in the knockdown embryos may also reflect redundant or parallel pathways

that work in conjunction with Bhlhb5 to direct interneuron differentiation. Indeed, the initial pattern of HD proteins expressed by spinal cord progenitors and formation of discrete progenitor domains along the dorsoventral axis were not significantly altered by the loss of Bhlhb5. In addition, we have observed that the closely related gene *Bhlhb4* partially overlaps the expression of *Bhlhb5* in the spinal cord, particularly in the mouse, and the combined misexpression of Bhlhb4 and Ngn2 in the chick can induce ectopic V2a formation much like Bhlhb5 and Ngn2 (K.S. and B.G.N, unpublished data). *Bhlhb5* mutant mice have recently been described, and although these animals display defects in neuronal differentiation in the retina and cortex (Feng et al., 2006; Joshi et al., 2008) and neuronal survival in some dorsal spinal interneurons (Ross et al., 2010), they have not been reported to exhibit the motor coordination phenotype that might be expected if ventral interneuron development were impaired. The generation of *Bhlhb4; Bhlhb5* double knockouts may thus be required to reveal the function of these genes in spinal interneuron development. It is further possible that the chick spinal cord is inherently more sensitive to Bhlhb5 loss, as Bhlhb5 appears to be more extensively expressed from the earliest stages of interneuron progenitor formation than that seen in mice, where Bhlhb5 is more highly associated with postmitotic neurons.

Bhlhb5 and the spatial organization of neurogenesis in the spinal cord

In addition to its ability to direct specific interneuron fates, Bhlhb5 appears to play an equally important role in establishing the domain-restricted pattern of Notch ligands and Fringe proteins in the spinal cord. Bhlhb5 is closely associated with the expression of Jagged1 in both pdI6 and p1 progenitors, and Dll4 in V2a interneurons.

Neighboring progenitors lacking Bhlhb5 express a different Notch ligand, Dll1, and the Notch regulator Lfng. When Bhlhb5 function was reduced, the domains of Jagged1 and *Dll4* expression were diminished while Dll1 and Lfng domains expanded. It is currently unclear whether these changes reflect a positive effect of Bhlhb5 on Jagged1 and *Dll4* expression or an inhibitory effect on Dll1 and Lfng, though the latter seems more likely given that Bhlhb5 appears to carry out most of its functions as a transcriptional repressor. In either case, these results provide novel insights into the mechanisms through which the alternating pattern of Notch ligands and Fringe genes in the spinal cord is established.

How does the spatial organization of Notch ligands and Fringe proteins contribute to spinal cord development? Each of the progenitor domains along the dorsoventral axis appears to differentiate on a characteristic schedule, and the focal expression of different Notch ligands may play an important role in guiding this process. In principle, the domain restricted expression of different Notch ligands could be used to enable cell-cell communication between neighboring progenitor domains to coordinate the timing of their differentiation and the balanced production of specific cell types. An example of this activity has been observed in the zebrafish spinal cord where the localized expression of Jagged2 within the pMN is critical for the generation of KA neurons from the neighboring p3 domain (Yeo and Chitnis, 2007).

In our Bhlhb5 knockdown experiments, we similarly found that the loss of Jagged1 in dI6 and p1 progenitors coincided with the reduced generation of V0 interneurons that normally lie adjacent to Bhlhb5^{+} cells, raising the possibility that Jagged1 might provide a positive stimulus for V0 generation. However, this interpretation is inconsistent with recent analysis of spinal cords in which either Jagged1

or Dll1 have been genetically inactivated or misexpressed which found that these manipulations only affect the differentiation behavior of the cells that normally express each ligand (Marklund et al., 2010). Hence, cells that express Jagged1 are only sensitive to changes in Jagged1 levels and unaffected by changes in Dll1, and vice versa. These differential sensitivities have been attributed to the domain-restricted expression of Lfng and Manic Fringe which have the ability to potentiate Dll1 signaling while simultaneously blocking Jagged1 signaling (D'Souza et al., 2008; Marklund et al., 2010).

In reconciling our results with those of Marklund et al. (2010), it is notable that in both our Bhlhb5 misexpression and knockdown experiments the expression of both Notch ligands and Fringe proteins are disrupted. Under these conditions, the breakdown in Fringe proteins might impair the ability of progenitors to discriminate between different Notch ligands and thus inappropriately respond to signals produced by their neighbors. Likwise, the expansion of Dll1 seen in the Bhlhb5 knockdowns could alter Notch signaling and the differentiation behavior of Bhlhb5-negative progenitors that are normally sensitive to this ligand. Our results are thus not incompatible, and together support a model in which Bhlhb5 plays a critical role in ensuring that both Notch ligands and Fringe proteins are confined to spatially restricted compartments to allow Notch signaling to operate selectively within a given progenitor group without affecting neighboring cells (Fig. 2.8B).

Moreover, the differential expression of Notch ligands and Fringe proteins may further act to preserve or reinforce progenitor domain borders. Notch signaling plays an important role in the dorsoventral compartmentalization of the *Drosophila* wing (Major and Irvine, 2005), as well as in the formation of boundary cells in the vertebrate central

nervous system (Baek et al., 2006). In this way, the differential expression of Jagged1 and Dll1/Lfng mediated by Bhlhb5 could help prevent the mixing of progenitors to maintain the dorsoventral positions from which neurons and glia emerge over time.

Bhlhb5 as an upstream regulator of Dll4-Notch signaling and V2 interneuron diversity

Once neural progenitor domains are established, multiple classes of neurons and glial cells emerge from seemingly homogeneous progenitor populations. Recent studies of V2 interneuron development have provided important insights into the molecular mechanisms through which this diversity may be achieved. The p2 progenitor population produces two functionally distinct classes, excitatory V2a neurons and inhibitory V2b neurons, using cell-cell signaling events mediated by Dll4 and Notch receptors (Del Barrio et al., 2007; Peng et al., 2007). The differential activity of Notch signaling between neighboring cells leads to the assembly of opposing transcription factor complexes that specify the type of V2 interneuron formed: cells that express Bhlhb5 and high levels of Dll4 form V2a neurons while the cells receiving high levels of Notch signaling express Foxn4 and Scl, and consequently adopt the V2b fate (Del Barrio et al., 2007; Peng et al., 2007).

This break in symmetry could in principle occur by a stochastic imbalance in the expression of Dll4 and Notch receptors within all p2 progenitors akin to the lateral inhibition model associated with *Drosophila* neuroblast formation (Skeath and Thor, 2003). Alternatively, it could occur in a more deterministic manner, through the concerted actions of Bhlhb5 in establishing the pattern of *Dll4* expression specifically within V2a neurons to elicit V2b formation. Our data are most consistent with the latter

possibility, considering that Bhlhb5 is detectable in p2 progenitors before the onset of the V2a/V2b subdivision, and that the coexpression of Bhlhb5 alone and in combination with Ngn2 is sufficient to induce V2a formation and *Dll4* expression throughout the spinal cord. Moreover, knockdown of endogenous Bhlhb5 function reduced both V2a interneuron generation and *Dll4*, and produced a loss rather than gain in V2b neuron formation. These results lead us to propose that V2 interneuron diversity emerges through a hierarchical relationship between Bhlhb5 and Notch signaling. Bhlhb5 promotes V2a interneuron differentiation leading to *Dll4* expression and the formation of V2b neurons in neighboring cells (Fig. 2.8C). In this way, Bhlhb5 provides a critical function in translating the early patterning information bestowed by morphogen signals into local Notch signaling events that both extend neural diversity and ensure that the formation of excitatory and inhibitory neurons is coupled for the coordinated control of motor functions.

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Chapter 2 Notes

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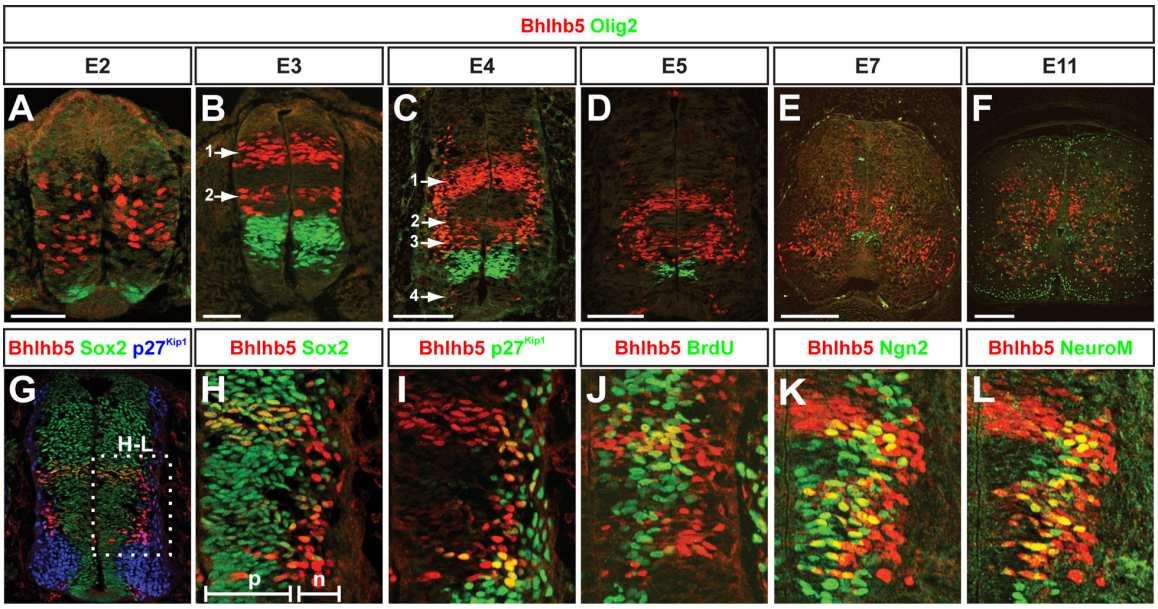


Figure 2.1. Bhlhb5 is complementary to Olig2 in spinal cord progenitors and retained by subsets of differentiating neurons.

(A-F) Antibody costaining analysis of Bhlhb5 (red) and Olig2 (green) expression in the developing chick spinal cord, from E2 (HH stage 10) to E11 (HH stage 37). Arrows and numbers in panels B and C indicate the appearance of distinct stripes of Bhlhb5 expression in neural progenitors. Scale bars = 50 µm in A-B; 100 µm in C-D, and 500 µm in E-F.

(G-I) At E4 (HH stage 23), Bhlhb5 is expressed by both $Sox2^{+}$ neural progenitors (p) and postmitotic neurons (n) that express the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

(J) $Bhlhb5^{+}$ progenitors are labeled by a 30-minute pulse of BrdU prior to embryo collection.

(K-L) Bhlhb5 transiently overlaps with the proneural bHLH proteins Ngn2 and NeuroM.

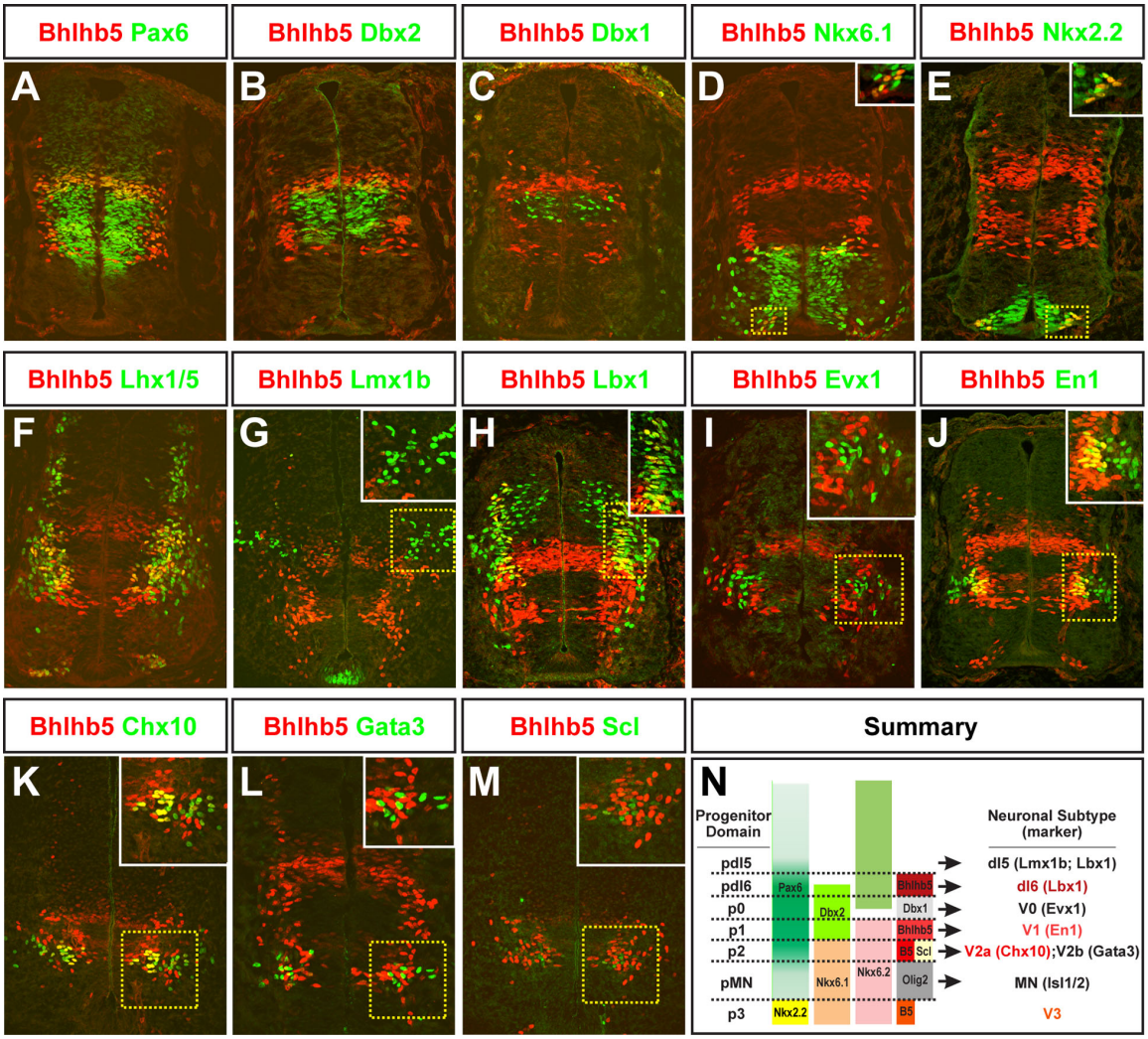


Figure 2.2. Bhlhb5 is selectively expressed by dI6, V1, and V2a progenitors and their interneuron progeny.

(A-E) Antibody costaining of Bhlhb5 (red) and homeodomain proteins (green) that demarcate the boundaries of progenitor domains in the E4 chick spinal cord. Insets in the panels show enlargements of the indicated regions.

(F-M) At E5, Bhlhb5 is expressed by several distinct groups of Lhx1/5^{+} interneurons identified by their patterns of transcription factor expression. (N) Summary of the expression pattern of Bhlhb5 and its association with specific classes of developing interneurons.

Figure 2.3. Bhlhb5 depends on retinoid signaling and Pax6 activity, and is spatially restricted by cross-repressive interactions with Olig2 and Dbx1.

(A-D) Misexpression of a dominant-negative RA receptor (dnRAR) represses both Pax6 and Bhlhb5. Bhlhb5 is restored by coelectroporation of a Pax6 expression construct. '+' indicates the transfected side of the spinal cord.

(E-F) Bhlhb5 is lost in E10.5 *Pax6*$^{Sey/Sey}$ mutant mice.

(G-H) Misexpression of Olig2 in chick represses Bhlhb5.

(I-J) Bhlhb5 expression expands ventrally in E10.5 *Olig2* mutant mice.

(K-L) Bhlhb5 expression expands into the p0 progenitor domain marked by LacZ expression in E11.5 *Dbx1*$^{LacZ/LacZ}$ mutant mice while *Dbx1*$^{LacZ/+}$ littermate controls show limited overlap.

(M-Q) Misexpression of a Myc-tagged form of Bhlhb5 in chick does not alter Pax6 but suppresses Dbx1 and to a lesser extent Olig2. Nkx6.1 expression appears to shift dorsally due to a mild suppressive effect of Bhlhb5 on Dbx2 (data not shown). Images are representative of >5 embryos for each experimental condition above.

(R) Summary of the upstream signaling pathways and cross-repressive interactions that regulate Bhlhb5 expression in the spinal cord.

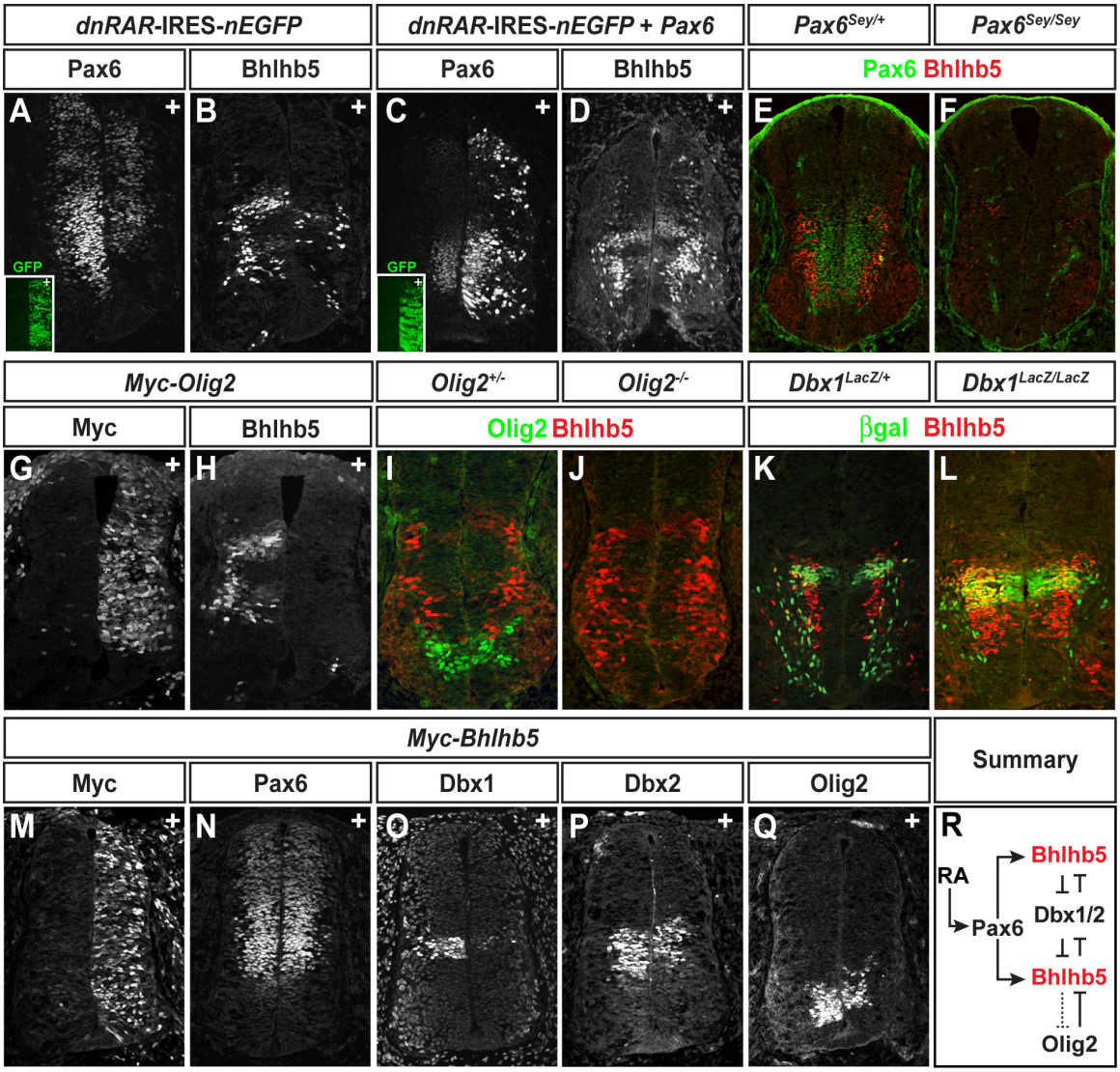

dnRAR-IRES-nEGFP
dnRAR-IRES-nEGFP + Pax6
Pax6Sey/+
Pax6Sey/Sey
Pax6
Bhlhb5
Pax6 Bhlhb5
A
B
C
D
E
F
GFP
Myc-Olig2
Olig2+/-
Olig2-/-
Dbx1LacZ/+
Dbx1LacZ/LacZ
Myc
Olig2 Bhlhb5
βgal Bhlhb5
G
H
I
J
K
L
Myc-Bhlhb5
Summary
Dbx1
Dbx2
Olig2
M
N
O
P
Q
R
RA
Pax6
Dbx1/2

Figure 2.4. Misexpression of Bhlhb5 and Ngns leads to ectopic generation of dI6, V1, and V2a interneurons and the suppression of other interneuron classes.

(A-E) Bhlhb5 misexpression mildly expands $Lhx3^+$ and $Chx10^+$ V2a interneuron formation (brackets), and reduces $Gata3^+$ and Scl^+ V2b interneurons. '+' indicates the transfected side of the spinal cord.

(F-J) Ngn2 misexpression promotes neuronal differentiation, but does not alter V2a or V2b fates.

(K-S) The combined misexpression of Bhlhb5 with Ngn2 produces ectopic $Lhx3^+$ and $Chx10^+$ V2a interneurons (L-M) throughout the spinal cord (brackets) and $Lbx1^+$ cells in the ventral spinal cord (Q). This manipulation simultaneously decreases the endogenous formation of dI4-6 (P-Q), V1 (S), and V2b interneurons (N-O). Note that the formation of $Lbx1^+$ $Lmx1b^+$ dI5 interneurons is also potently suppressed by Ngn2 alone (P,Q; see also Figure 2.12 S4D,K). All images shown are representative of >10 embryos examined for each experimental condition.

(T-W) Quantification of neurons formed under the electroporation conditions described above. Total cell numbers are represented as % control based on the ratio of cell numbers on the experimental side of the spinal cord compared to the non-electroporated, contralateral side. Control electroporations refers to embryos transfected with an empty expression vector. Ectopic En1 and Chx10 cells were defined as cells dorsal or ventral to their normal position on the contralateral control side of the spinal cord. Ventral Lbx1 cells were scored as the number of Lbx1 cells below the midpoint of the dorsoventral axis.

Means ± s.e.m. plotted are representative of multiple sections collected from >10 successfully electroporated embryos for each experimental condition tested. Statistical significance between experimental and control conditions was determined by Student's t-test: * $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

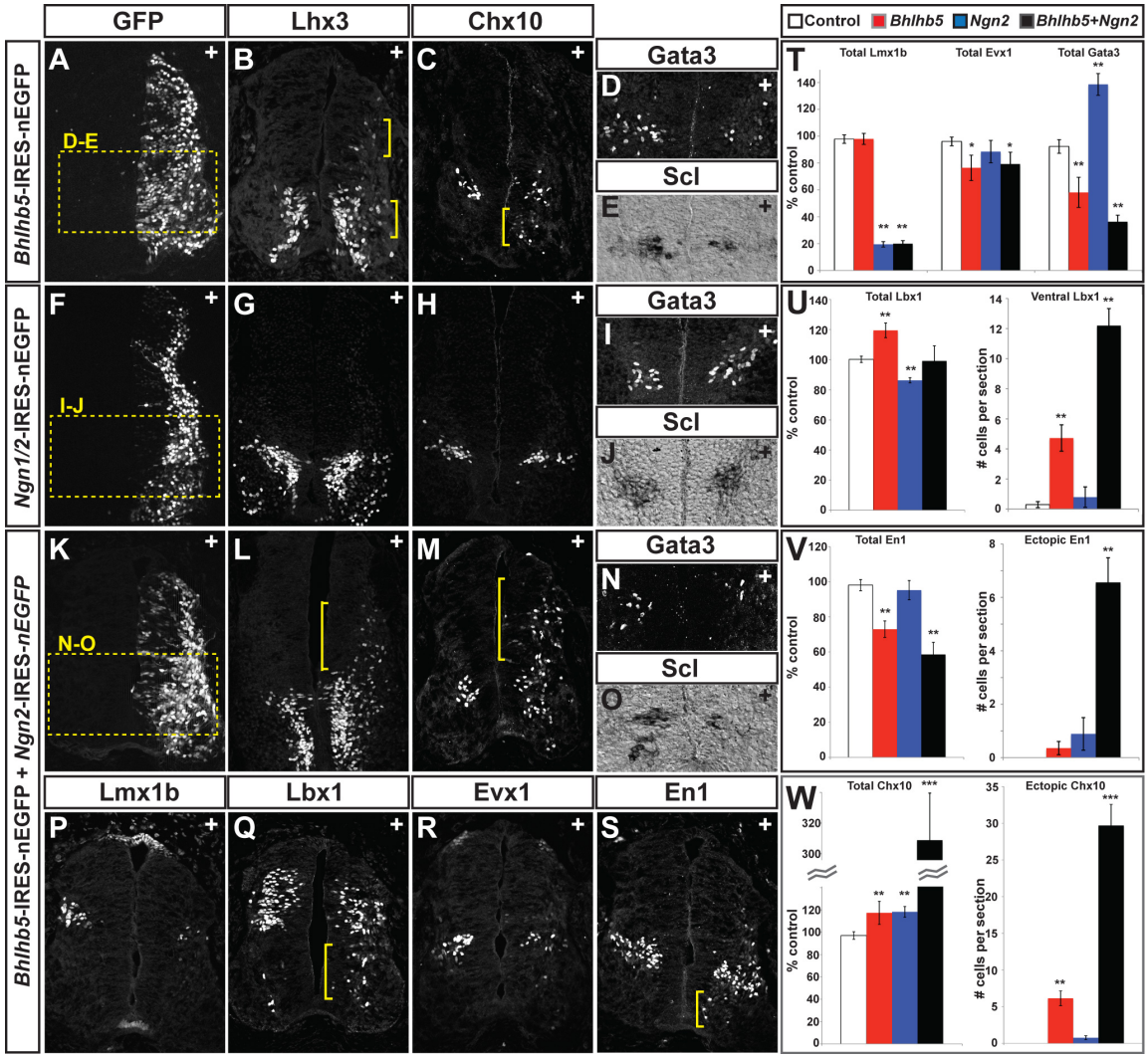

GFP
Lhx3
Chx10
Gata3
Scl
Lmx1b
Lbx1
Evx1
En1
Bhlhb5-IRES-nEGFP
Ngn1/2-IRES-nEGFP
Bhlhb5-IRES-nEGFP + Ngn2-IRES-nEGFP
D-E
I-J
N-O
Control
Bhlhb5
Ngn2
Bhlhb5+Ngn2
Total Lmx1b
Total Evx1
Total Gata3
Total Lbx1
Ventral Lbx1
Total En1
Ectopic En1
Total Chx10
Ectopic Chx10
% control
# cells per section

Figure 2.5. Bhlhb5 directs specific neuronal fates through its actions as a transcriptional repressor.

(A) Schematic illustration of the modified forms of Bhlhb5 used to test transcriptional activity.

(B-P) Coexpression of Bhlhb5 bHLH-EnR and Ngn2 recapitulates the activities of full-length Bhlhb5 and Ngn2 while the equivalent coexpression of Bhlhb5 bHLH-VP16 or Bhlhb5$^{\Delta b}$ and Ngn2 has little effect on neuronal fates. '+' Indicates transfected side of the spinal cord in all cases. Results are representative of 5-10 embryos for each experimental condition.

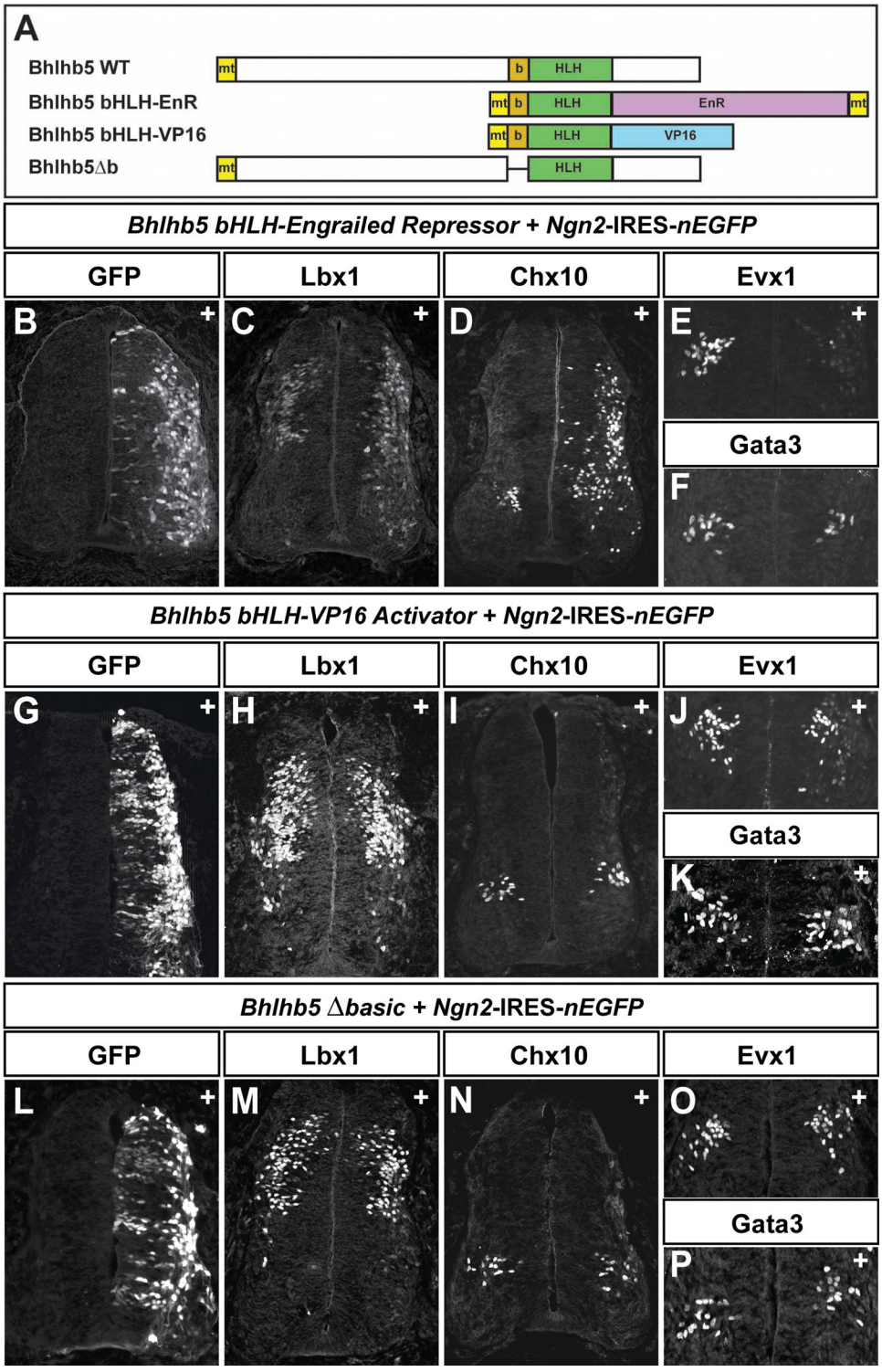

A
Bhlhb5 WT
mt
b
HLH
Bhlhb5 bHLH-EnR
EnR
Bhlhb5 bHLH-VP16
VP16
Bhlhb5Δb
Bhlhb5 bHLH-Engrailed Repressor + Ngn2-IRES-nEGFP
GFP
Lbx1
Chx10
Evx1
Gata3
B
C
D
E
F
Bhlhb5 bHLH-VP16 Activator + Ngn2-IRES-nEGFP
G
H
I
J
K
Bhlhb5 Δbasic + Ngn2-IRES-nEGFP
L
M
N
O
P

Figure 2.6. Loss of Bhlhb5 function reduces the formation of dI6, V1, and V2a interneurons as well as adjacent interneuron populations.

(A) Schematic showing the location of the three short hairpin targets with respect to the Bhlhb5 coding sequence.

(B-J) Antibody staining analysis of chick spinal cords electroporated at E3 with a cocktail of vectors producing 3 different short-hairpin RNAs (shRNA) against the endogenous *Bhlhb5* transcript.

(K-S) The equivalent electroporation with the cocktail of scrambled short-hairpin RNAs has no effect on numbers of cells expressing interneuron markers. '+' Indicates transfected side of the spinal cord in all cases. Images shown are representative of >10 embryos examined for each experimental condition.

(T) Chart displays the number of cells expressing each neuronal subtype marker expressed as a percentage of that seen on the contralateral, unelectroporated control side of the spinal cord. Means ± s.e.m. plotted are representative of multiple sections collected from >10 successfully electroporated embryos for each experimental condition tested. Statistical significance between experimental and control conditions was determined by Student's t-test: * $p < 0.05$; ** $p < 0.01$; ns, not significant.

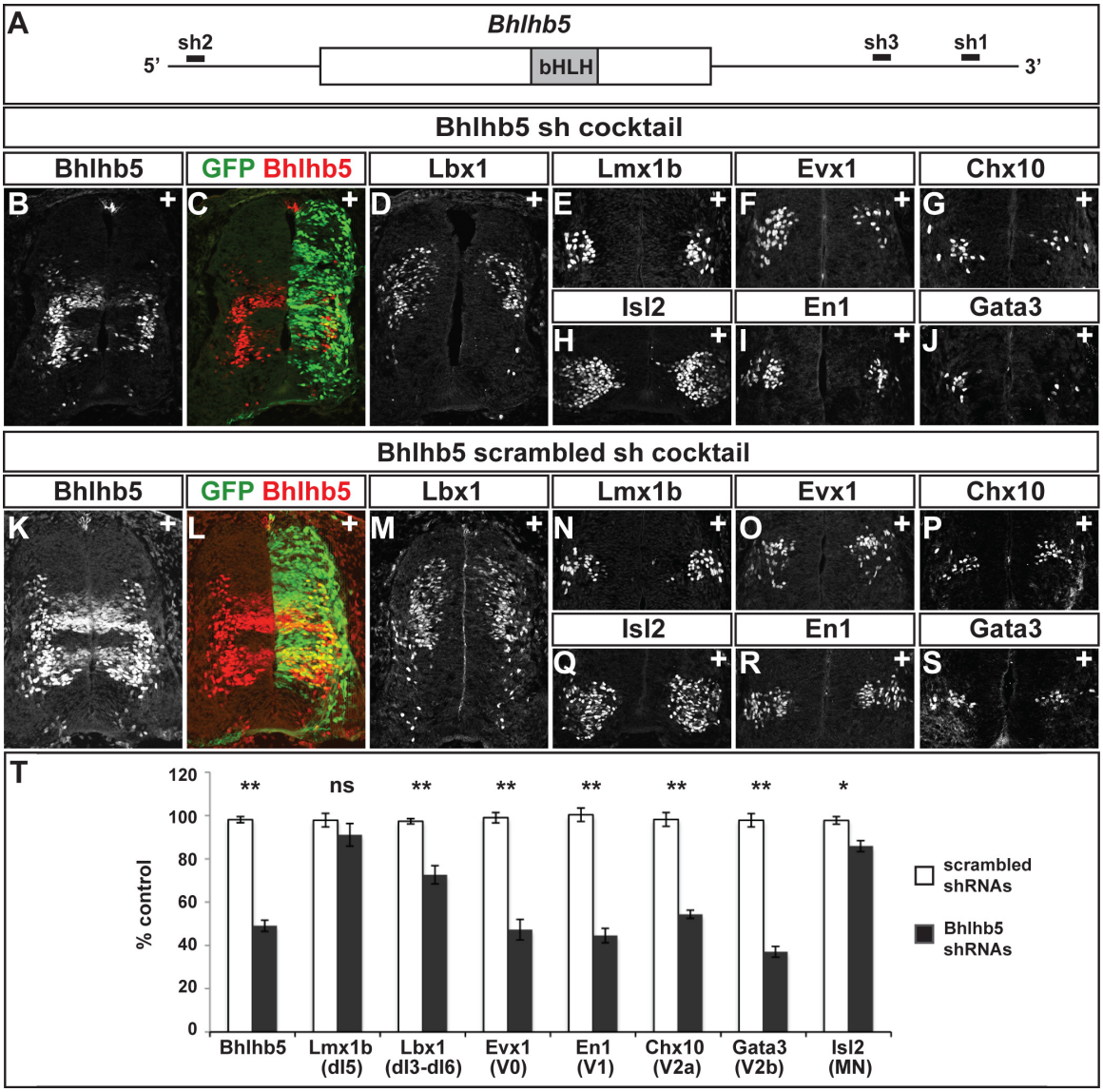
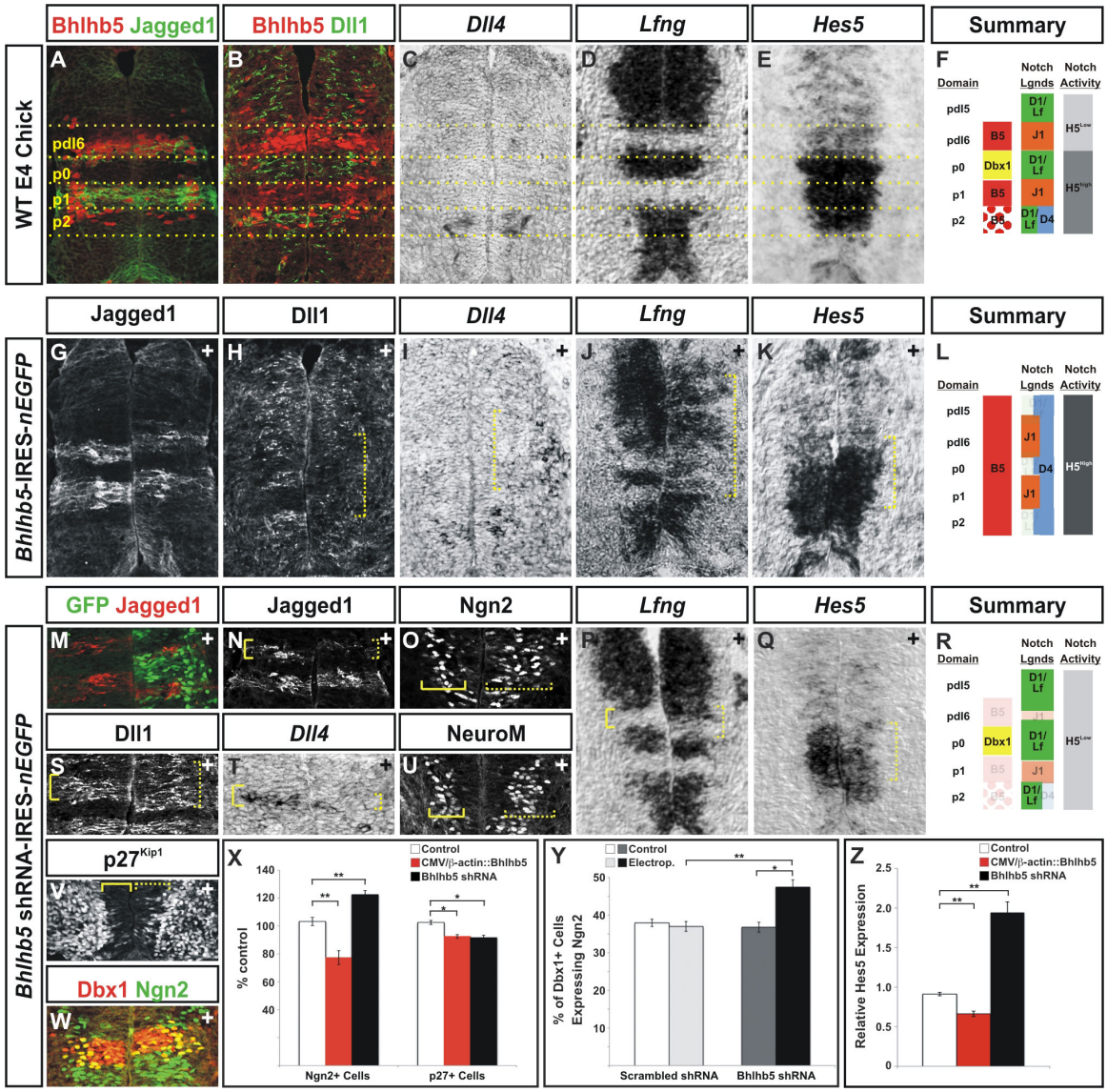

A
Bhlhb5
sh2
5'
bHLH
sh3
sh1
3'
Bhlhb5 sh cocktail
Bhlhb5
GFP Bhlhb5
Lbx1
Lmx1b
Evx1
Chx10
B
C
D
E
F
G
Isl2
En1
Gata3
H
I
J
Bhlhb5 scrambled sh cocktail
Bhlhb5
GFP Bhlhb5
Lbx1
Lmx1b
Evx1
Chx10
K
L
M
N
O
P
Isl2
En1
Gata3
Q
R
S
T
% control
120
100
80
60
40
20
0
** ns ** ** ** ** ** *
Bhlhb5
Lmx1b (dl5)
Lbx1 (dl3-dl6)
Evx1 (V0)
En1 (V1)
Chx10 (V2a)
Gata3 (V2b)
Isl2 (MN)
scrambled shRNAs
Bhlhb5 shRNAs

Figure 2.7. Bhlhb5 regulates the pattern of Notch ligands, *Lfng,* and neurogenesis in the spinal cord.

(A-D) Notch ligands and *Lfng* are expressed in a domain-restricted manner that mirrors that the spatially confined pattern of Bhlhb5. Jagged1 and *Dll4* are found in regions where Bhlhb5 is present, while Dll1 and *Lfng* are in regions where it is absent. (E) *Hes5* mRNA provides a readout for ongoing Notch pathway activity in the intermediate spinal cord. (F) Summary of the domain relationship between Bhlhb5, HD transcription factors, Notch ligands, and Notch pathway activity under control conditions. (G-L) Misexpression of Bhlhb5 represses both Dll1 and Lfng, and expands the domains of Jagged1 and Dll4. Under these conditions, *Hes5* levels appear to increase in intensity on the transfected side and expand dorsally. (M-V) Bhlhb5 knockdown leads to a reduction in Jagged1 and *Dll4*, and a concomitant expansion in Dll1 and Lfng, particularly in the pdI6 region. *Hes5* levels in the intermediate spinal cord decline and coincide with an increased density of differentiating neurons expressing Ngn2, NeuroM, and $p27^{Kip1}$. (W) While Bhlhb5 knockdown does not inhibit the formation of $Dbx1^{+}$ p0 cells, Ngn2 expression is nevertheless increased in this region. '+' Indicates transfected side of the spinal cord in all cases. Images shown are representative of > 5 embryos examined for each experimental condition. (X) Quantification of the effects of Bhlhb5 misexpression and knockdown on Ngn2 expression and the formation of $p27^{Kip1+}$ neurons. Experimental manipulations were compared to cells expressing the indicated markers on the contralateral control side of the spinal cord. An additional empty expression vector control was performed to account for effects of the electroporation procedure. Means ± s.e.m. are representative of multiple sections collected from >10 successfully electroporated embryos for each experimental condition tested and plotted as % of control. Statistical significance of the effects was determined by Student's t-test: * $p < 0.02$; ** $p < 0.002$. (Y) Comparison of the ratio of $Dbx1^{+}$ p0 cells expressing Ngn2 on the electroporated side of the spinal cord versus the contralateral control side. * $p < 0.001$ (paired samples); ** $p < 0.002$ (independent samples). (Z) Quantification of *Hes5* expression in the intermediate spinal cord electroporated with the indicated constructs relative to the contralateral control side. * $p < 0.02$; ** $p < 0.001$.


WT E4 Chick
Bhlhb5 Jagged1
Bhlhb5 Dll1
Dll4
Lfng
Hes5
Summary
A
B
C
D
E
F
pdl6
p0
p1
p2
Domain
Notch Lgnds
Notch Activity
pdl5
B5
Dbx1
J1
D1/ Lf
D4
H5Low
H5High
Bhlhb5-IRES-nEGFP
Jagged1
Dll1
G
H
I
J
K
L
GFP Jagged1
Ngn2
M
N
O
P
Q
R
Dll4
NeuroM
S
T
U
Bhlhb5 shRNA-IRES-nEGFP
p27Kip1
V
Dbx1 Ngn2
W
X
Control
CMV/β-actin::Bhlhb5
Bhlhb5 shRNA
% control
Ngn2+ Cells
p27+ Cells
Y
Electrop.
% of Dbx1+ Cells Expressing Ngn2
Scrambled shRNA
Z
Relative Hes5 Expression

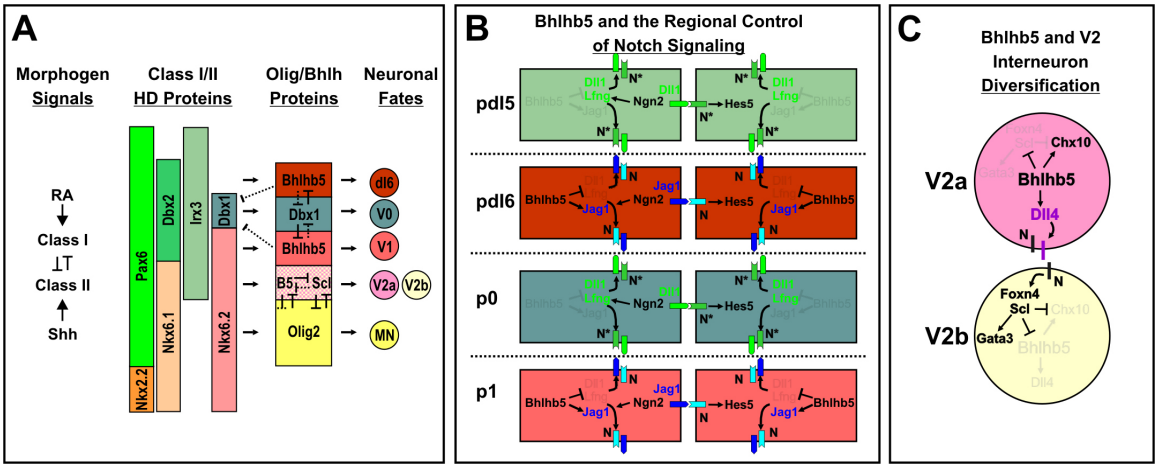


Figure 2.8. Consolidation of interneuron fate specification and differentiation through the actions of Bhlhb5 and the Notch signaling pathway.

(A) RA and Shh signaling respectively activate the expression of Class I and Class II HD proteins in the spinal cord, leading to the establishment of discrete progenitor domains. The combinatorial actions of these transcription factors, particularly Pax6, promotes the expression of Bhlhb5 in the intermediate spinal cord, which helps refine progenitor domain borders and drives the formation of dI6, V1, and V2 interneurons. Repressive interactions between Bhlhb5 and opposing fate determinants such as Scl subsequently divide V2 neurons into V2a and V2b subclasses. (B) Once progenitor domains are formed, Bhlhb5 regulates the expression of the Notch ligands Dll1 and Jagged1 along with Lfng. The differential expression of Lfng alters the ability of the Dll1 and Jagged1 to activate Notch receptors (N), thereby confining Notch signaling interactions within but not between progenitor domains (see Marklund et al., 2010 for more details).

(C) Within the V2 interneuron lineage, Bhlhb5 promotes V2a differentiation and the expression of Dll4. Dll4 in turn engages Notch receptors on neighboring V2 cells to induce Scl and Gata3, and thereby elicit V2b interneuron formation.

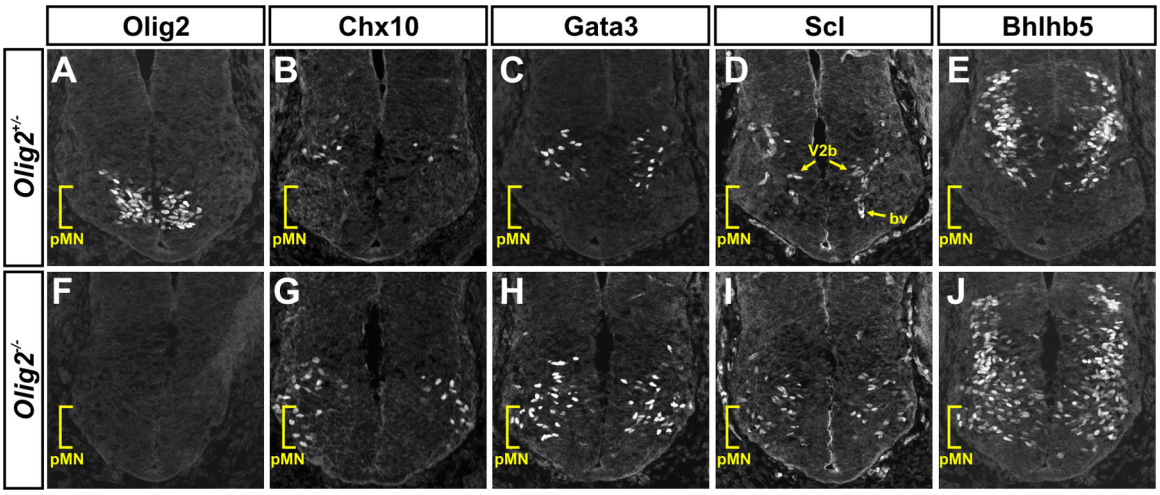


Figure 2.9. Supplementary Figure S1. Ventral expansion of Bhlhb5 expression coincides with ectopic interneuron formation in the *Olig2* mutant spinal cord.

(A-L) Antibody staining for Bhlhb5 and markers of ventral interneuron development in e10.5 *Olig2*$^{+/-}$ heterozygous control (A-E) and *Olig2*$^{-/-}$ homozygous mutant spinal cords (F-J). In controls, Bhlhb5 and markers of V2 interneuron development (Chx10, Gata3, and Scl) are confined to a position immediately dorsal to the Olig2^{+} pMN domain (indicated by brackets). In *Olig2* mutants, Bhlhb5 expression and V2 interneuron formation expand ventrally into the pMN region. In panel (D), Scl protein is detectable in both V2b interneurons and blood cells associated with blood vessels (bv) growing into the spinal cord.

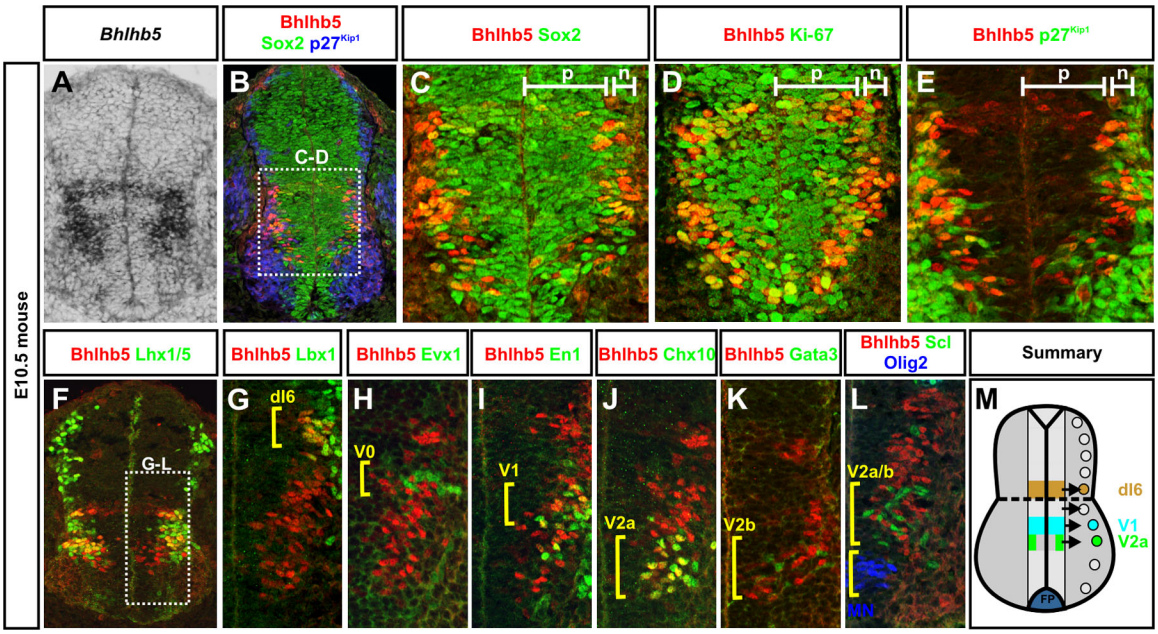


Figure 2.10. Supplementary Figure S2. Bhlhb5 is expressed by interneuron progenitors and subsets of newly differentiated interneurons in the developing mouse spinal cord.

(A-E) At E10.5, Bhlhb5 mRNA and protein expression are present in $Sox2^{+}$ and $Ki\text{-}67^{+}$ neural progenitors (p) in the ventricular zone as well as $p27^{Kip1+}$ neurons (n) lateral to the ventricular zone.

(F-L) Many $Bhlhb5^{+}$ neurons express the interneuron markers Lhx1/5 and markers characteristic of specific interneuron populations. G-L are enlargements of the area shown in F. Bhlhb5 is present in dI6 interneurons (Lbx1, G), V1 interneurons (En1, I), and V2a interneurons (Chx10, J), but absent in V0 interneurons (Evx1, H), V2b interneurons (Gata3 and Scl, K-L), and MN progenitors (Olig2, L) and differentiated MNs (data not shown).

(M) Schematic summary of Bhlhb5 expression in specific interneuron populations in the developing mouse spinal cord.

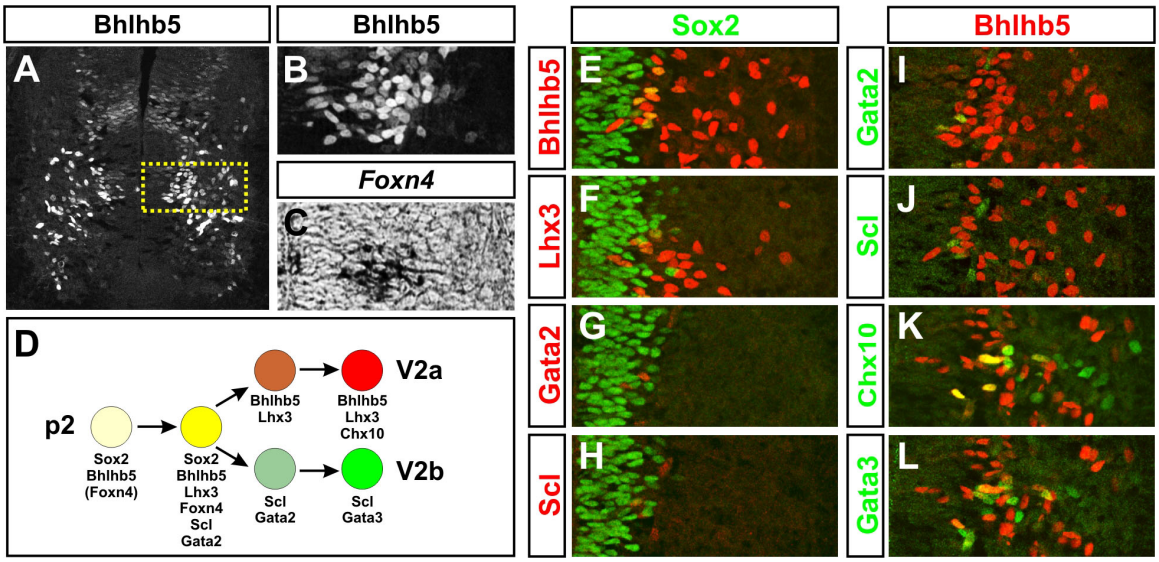


Figure 2.11. Supplementary Figure S3. Bhlhb5 is an early determinant of the V2 interneuron lineage.

(A) Expression of Bhlhb5 protein in the E5 chick spinal cord. Boxed region indicated is analyzed in greater detail in panels B,C,E-L.

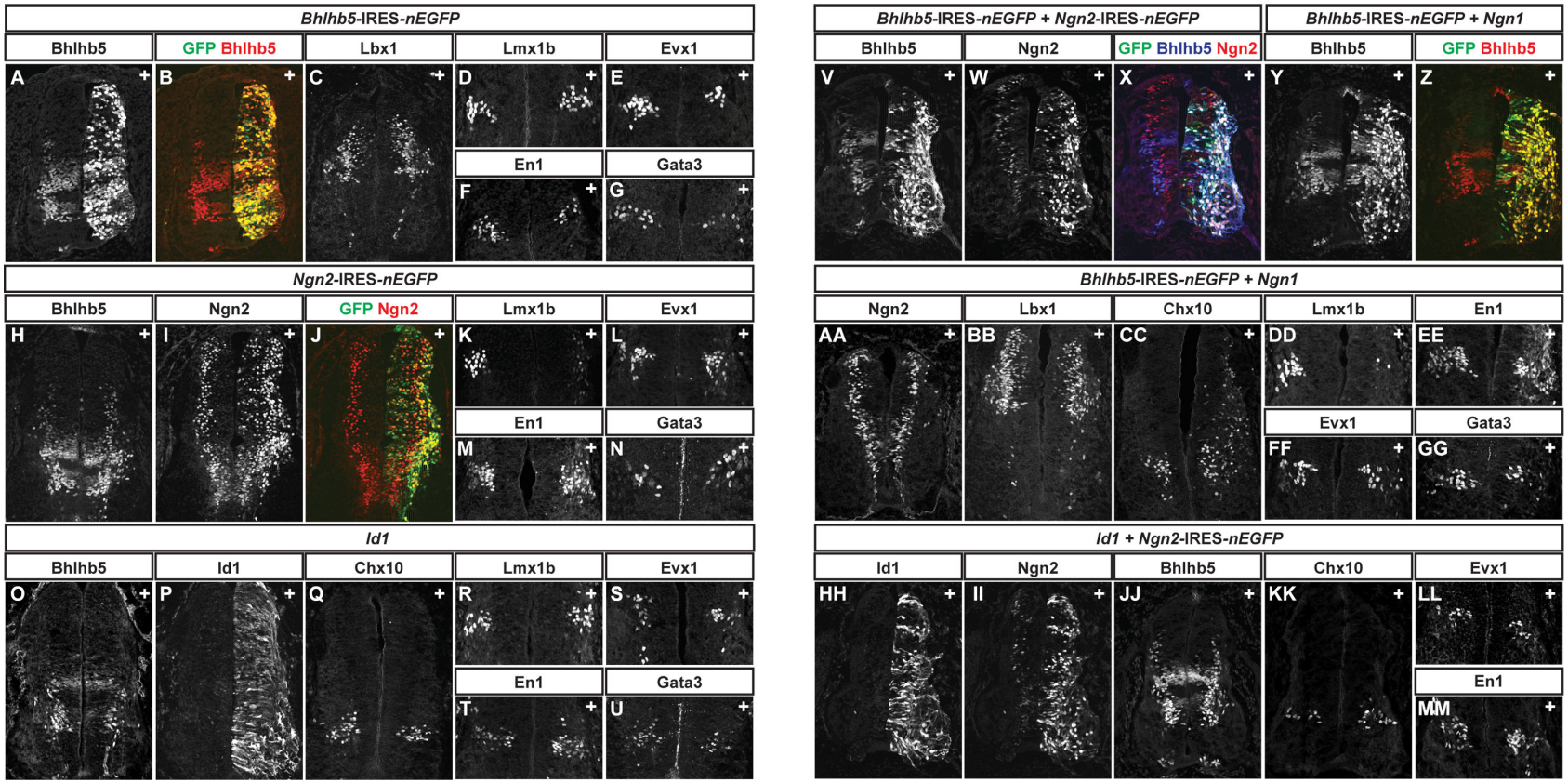
(B,C,E) Bhlhb5 protein expression first comes on in a subset of $Sox2^{+}$ neural progenitors and medial to those expressing *Foxn4*.

(D) Schematic summary of Bhlhb5 expression relative to previously characterized transcription factors associated with the bifurcation of p2 progenitors into V2a and V2b interneurons, based on costaining analysis shown in panels B,C,E-L.

(F-H) Lhx3, Gata2, and Scl show a much more limited overlap with Sox2 compared to Bhlhb5.

(I-L) Bhlhb5 expression transiently coexists with Gata2 and to a lesser extent Scl, and then becomes associated with $Chx10^{+}$ V2a interneurons rather than $Gata3^{+}$ V2b interneurons.

Figure 2.12. Supplementary Figure S4. The actions of Bhlhb5 in interneuron fate specification are distinct from that exhibited by Neurogenins and Id proteins.
(A-G) Misexpression of Bhlhb5 alone does not suppress the formation of $Lmx1b^{+}/Lbx1^{+}$ dI5 (C-D) interneurons, although the total number of the ventral-most $Lbx1^{+}$ interneurons (presumably dI6) was increased by ~20% (see Figure 2.4T). $Evx1^{+}$ V0 (E) and $En1^{+}$ V1 interneurons (F) were reduced by ~25%, while $Gata3^{+}$ V2b (G) interneurons were reduced by ~40% (see Figure 2.4U,V). These results indicate that Bhlhb5 misexpression alone broadly inhibits neuronal differentiation. These effects are likely to result from the suppressive effects of Bhlhb5 misexpression on Ngn2 expression and the overall generation of $p27^{Kip1+}$ postmitotic neurons (see Figure 2.7X).
(H-N) Misexpression of Ngn2 alone increases the total number of interneurons formed, but does not appear to influence the generation of any particular neuronal subtype types with the exception of $Lmx1b^{+}$ dI5 (K) interneurons, which were potently suppressed.
(O-U) Misexpression of the bHLH inhibitor Id1 alone slightly decreases the number of interneurons formed but does not affect the specification of particular ventral interneuron subtypes. (V-Z) Antibody costaining analysis shows that cells coelectroporated with Bhlhb5 and Ngn1 or Ngn2 expression vectors express these proteins along with the GFP transfection marker. (AA-GG) The combined misexpression of Bhlhb5 and Ngn1 leads to the ectopic generation of $Lbx1^{+}$ dI6 interneurons (BB) and $Chx10^{+}$ V2a interneurons (CC), and reduces the formation of $Gata3^{+}$ V2b interneurons (GG). A slight reduction in numbers of $Evx1^{+}$ V0 (FF) and $En1^{+}$ V1 (EE) interneurons was also observed. These actions are nearly identical to that observed with co-expression of Bhlhb5 and Ngn2 vectors (Figure 2.4K-S). As with Ngn2, Ngn1 misexpression alone or in combination with Bhlhb5 suppresses $Lmx1b^{+}$ dI5 interneuron formation (DD). (HH-MM) The combined misexpression of Ngn2 and Id1 does not alter Bhlhb5 expression (JJ), or lead to dramatic changes in the dorsoventral positioning of interneuron subtypes as seen with Bhlhb5 and Ngn2 coexpression (KK-MM, compare with Figure 2.4K-S). These results indicate that the actions of Bhlhb5 in interneuron fate specification are distinct from the inhibitory actions of Id proteins.


Bhlhb5-IRES-nEGFP
Bhlhb5
GFP Bhlhb5
Lbx1
Lmx1b
Evx1
A
B
C
D
E
En1
Gata3
F
G
Ngn2-IRES-nEGFP
Bhlhb5
Ngn2
GFP Ngn2
Lmx1b
Evx1
H
I
J
K
L
En1
Gata3
M
N
Id1
Bhlhb5
Id1
Chx10
Lmx1b
Evx1
O
P
Q
R
S
En1
Gata3
T
U
Bhlhb5-IRES-nEGFP + Ngn2-IRES-nEGFP
Bhlhb5
Ngn2
GFP Bhlhb5 Ngn2
V
W
X
Bhlhb5-IRES-nEGFP + Ngn1
Bhlhb5
GFP Bhlhb5
Y
Z
Bhlhb5-IRES-nEGFP + Ngn1
Ngn2
Lbx1
Chx10
Lmx1b
En1
AA
BB
CC
DD
EE
Evx1
Gata3
FF
GG
Id1 + Ngn2-IRES-nEGFP
Id1
Ngn2
Bhlhb5
Chx10
Evx1
HH
II
JJ
KK
LL
En1
MM

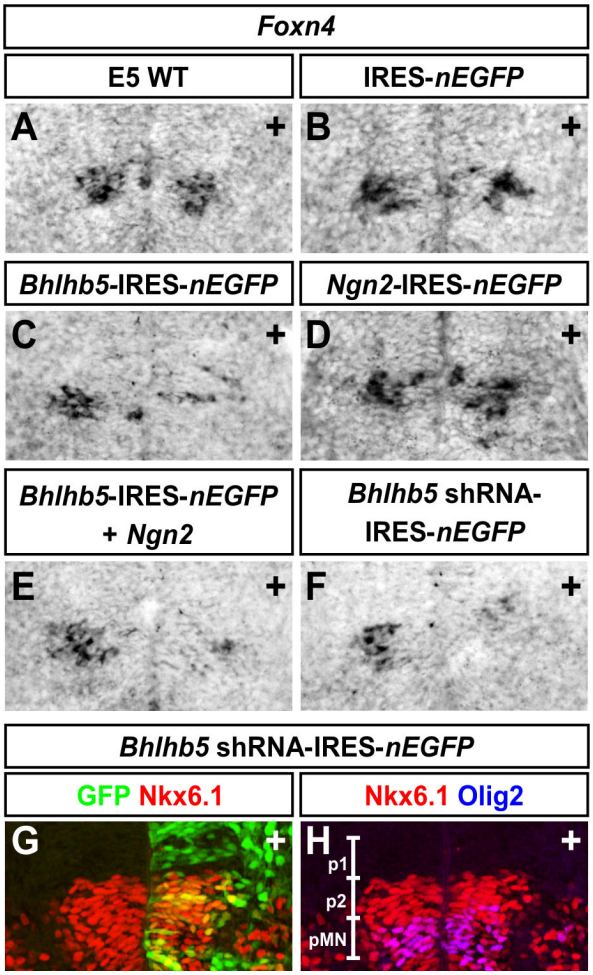


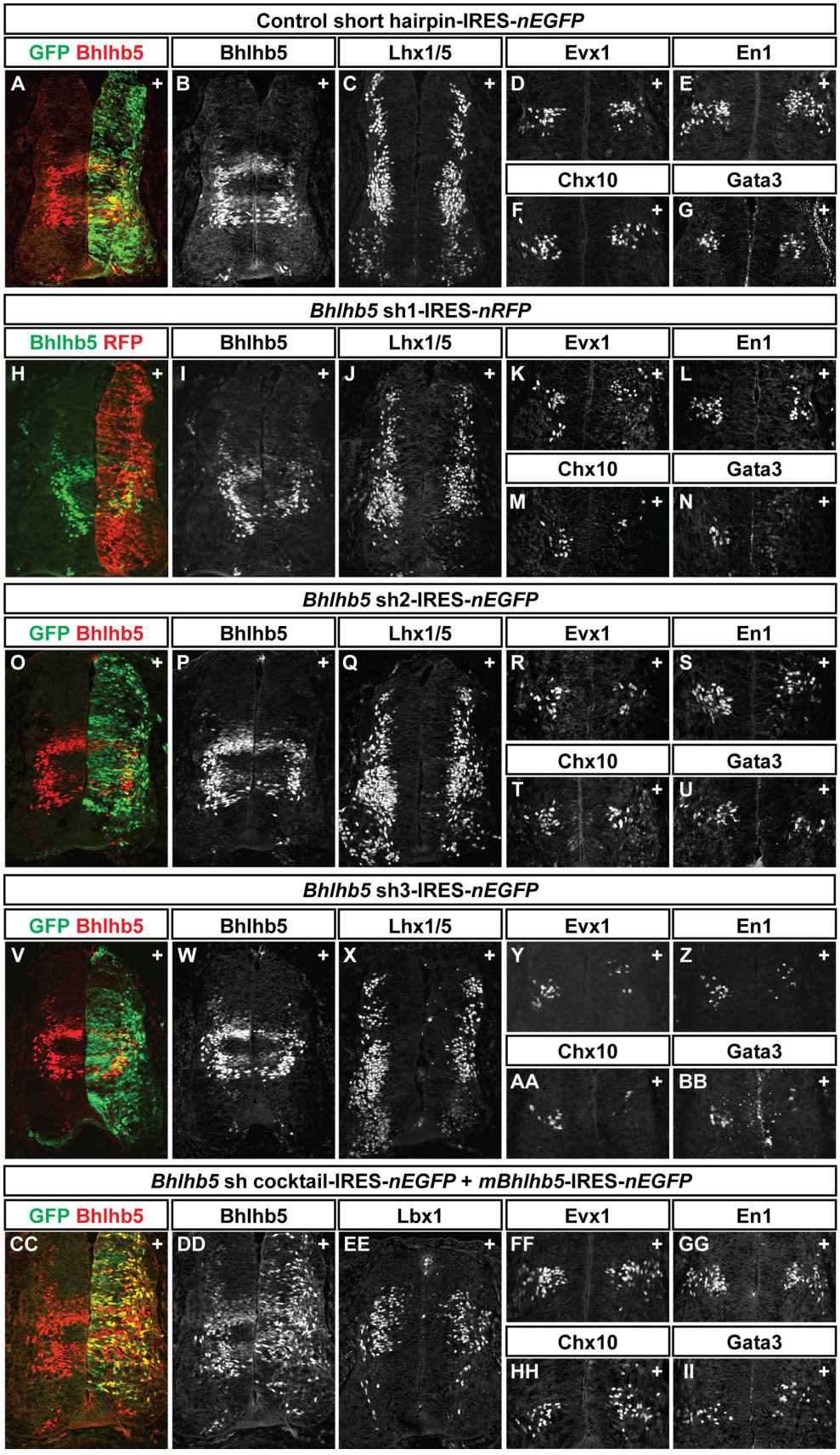
Figure 2.13. Supplementary Figure S5. Bhlhb5 misexpression alone or in combination with Ngn2 reduces expression of the early V2b determinant *Foxn4*. (A-B) *Foxn4* mRNA in wildtype and control electroporated spinal cords. '+' indicates the transfected side of the spinal cord in all cases. (C-E) Bhlhb5 misexpression alone or in combination with Ngn2 reduces *Foxn4* expression, in contrast to the slight expansion in *Foxn4* seen with Ngn2 misexpression alone. (F) shRNA-mediated knockdown of Bhlhb5 reduces Foxn4 expression in the same way that it reduces other V2b-associated markers. (G-H) Bhlhb5 knockdown does not alter the dorsoventral position of p2 progenitors marked by an Nkx6.1^{+} domain dorsal to Olig2^{+} pMN cells.

Figure 2.14. Supplementary Figure S6. Bhlhb5 knockdown effects are elicited by several different shRNA constructs and rescued by the coexpression of the mouse *Bhlhb5* gene.

(A-G) Antibody staining analyses of embryos electroporated with an shRNA vector containing a control short hairpin sequence show no disruption in numbers of ventral interneuron subtypes formed.

(H-BB) Embryos electroporated with plasmid vectors producing three different shRNAs that target the chick *Bhlhb5* mRNA have similar effects in reducing the level of Bhlhb5 protein expression (I,P,W). The loss of Bhlhb5 expression achieved with each of these short hairpin constructs coincided with a reduction in the appearance of Lhx1/5^{+} cells (J,Q,X), Evx1^{+} V0 interneurons (K,R,Y), En1^{+} V1 interneurons (L,S,Z), Chx10^{+} V2a interneurons (M,T,AA), and Gata3^{+} V2b interneurons (N,U,BB).

(CC-II) The suppressive effects of the cocktail of the three shRNAs against chick *Bhlhb5* (Figure 2.6) can be rescued by the coelectroporation of a mouse Bhlhb5 expression vector that lacks the shRNA target sites. Some ectopic Chx10^{+} V2a interneurons were seen under these conditions while other classes were repressed, consistent with the ability of misexpressed Bhlhb5 to promote the formation of these cells while suppressing other interneuron subtypes.


Control short hairpin-IRES-nEGFP
GFP Bhlhb5
Bhlhb5
Lhx1/5
Evx1
En1
A
B
C
D
E
Chx10
Gata3
F
G
Bhlhb5 sh1-IRES-nRFP
Bhlhb5 RFP
Bhlhb5
Lhx1/5
Evx1
En1
H
I
J
K
L
Chx10
Gata3
M
N
Bhlhb5 sh2-IRES-nEGFP
GFP Bhlhb5
Bhlhb5
Lhx1/5
Evx1
En1
O
P
Q
R
S
Chx10
Gata3
T
U
Bhlhb5 sh3-IRES-nEGFP
GFP Bhlhb5
Bhlhb5
Lhx1/5
Evx1
En1
V
W
X
Y
Z
Chx10
Gata3
AA
BB
Bhlhb5 sh cocktail-IRES-nEGFP + mBhlhb5-IRES-nEGFP
GFP Bhlhb5
Bhlhb5
Lbx1
Evx1
En1
CC
DD
EE
FF
GG
Chx10
Gata3
HH
II

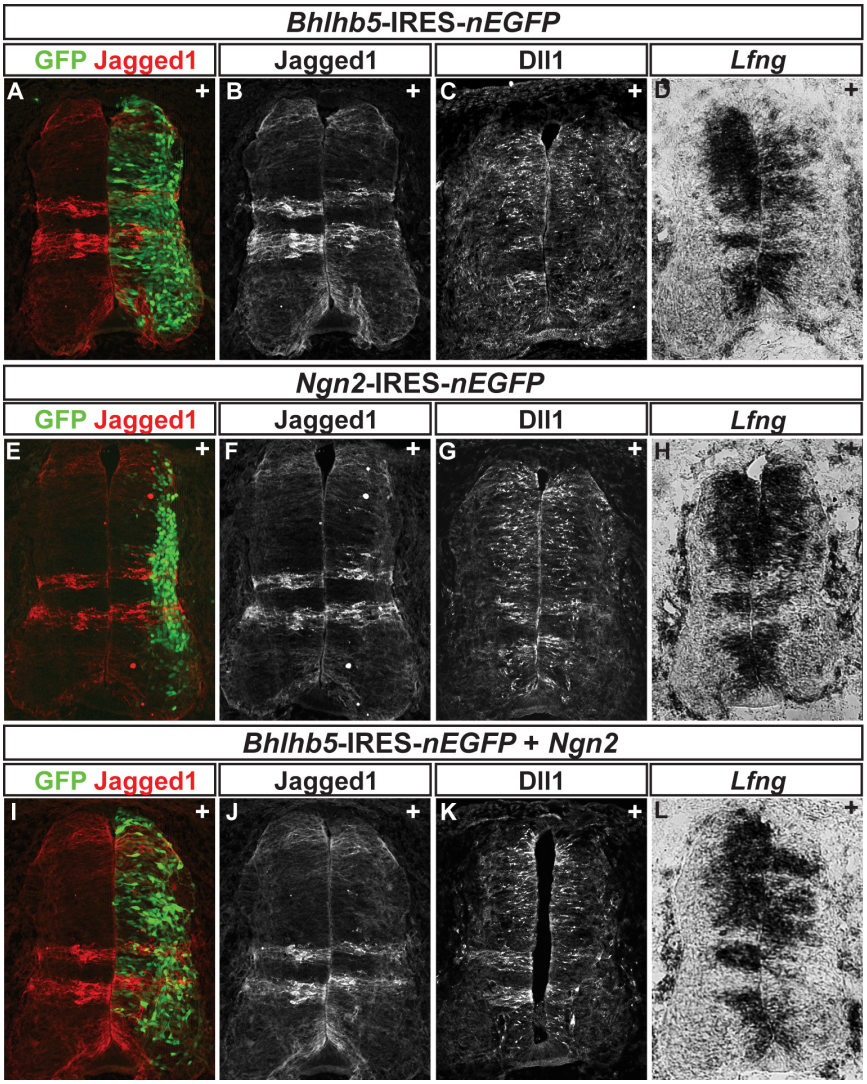


Figure 2.15. Supplementary Figure S7. Bhlhb5 misexpression alone or in combination with Ngn2 reduces Jagged1, Dll1 and *Lfng* expression.

(A-D) Misexpression of Ngn2 leads to small expansions in the pattern of Jagged1 (indicated by the bracket) but little change in Dll1 or *Lfng* .

(E-I) The combined misexpression of Bhlhb5 and Ngn2 reduces Jagged1, Dll1 and *Lfng*, in a manner similar to that seen with Bhlhb5 misexpression alone. *Dll4* is greatly expanded under these conditions.

'+' indicates the transfected side of the spinal cord in all panels.

Supplementary Table S1. Antibodies used for immunohistochemical analysis.

Host Species	Antigen (clone name)	Source and/or References
Goat	β-galactosidase	Biogenesis Inc., 4600-1409
Guinea pig and Rabbit	Chick Bhlhb5 amino acids 31-50: RSPPGLDLSHPRDRQPSPLAC	This study
Guinea pig	Mouse Bhlhb5 amino acids 47-63: APPTRERPASSSSPLGC	This study
Goat	Hamster Beta3/Bhlhb5	Santa Cruz Biotechnology, sc-6045
Rat	BrdU (BU1/75)	Accurate Chemical, MAS-250p
Rabbit	Chick Chx10	Ericson et al., 1997
Rabbit	Chick Dbx1	Pierani et al., 1999; Vue et al., 2007
Rabbit	Chick Dbx2	Pierani et al., 1999
Rabbit	Human Dll1	Santa Cruz Biotechnology, sc-9102
Goat	Mouse Dll4	R&D Systems, AF1389
Mouse	Chick En1 (4G11)	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Mouse Evx1 (99.1-3A2)	Developmental Studies Hybridoma Bank; see also Pierani et al., 1999
Mouse	Human Gata2	Santa Cruz Biotechnology, sc-267
Goat	Human Gata3	Santa Cruz Biotechnology, sc-1236

Sheep	Green Fluorescent Protein	Biogenesis, 4745-1051
Mouse	Rat Isl1 (39.4D5)	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Chick Isl2 (51.4H9)	Briscoe et al., 2000
Goat	Human Jagged1	Santa Cruz Biotechnology, sc-6011
Guinea Pig and Rabbit	Mouse Lbx1	Muller et al., 2005; Muller et al., 2002
Mouse	Chick Lhx3 (67.4E12)	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Rat Lhx5 (4F2)	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Chick Lmx1b (50.5A5)	Developmental Studies Hybridoma Bank
Guinea Pig and Rabbit	Myc epitope tag	Novitch et al., 2001
Rabbit and Rat	Chick NeuroM	Generous gift of Dr. Paul Trainor and Bylund et al., 2003
Rabbit	Human NF-1A	Active Motif, 39329
Guinea pig	Chick Ngn2 amino acids 194-211: CEHWPPPRGRFAPPPPPHR	This study; see also Sandberg et al., 2005
Rabbit	Chick Ngn2	Zhou et al., 2001
Mouse	Chick Nkx2.2 (74.5A5)	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Rat Nkx6.1 (F55A10)	Developmental Studies Hybridoma Bank

Rabbit	Chick Olig2	Novitch et al., 2001
Guinea pig	Mouse Olig2	Wichterle et al., 2002
Mouse	Human $p27^{Kip1}$	BD Biosciences, 610241
Mouse	Chick Pax6	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Mouse	Chick Pax7	Developmental Studies Hybridoma Bank; see also Briscoe et al., 2000; Ericson et al., 1997
Guinea pig	Chick Scl amino acids 5-19: RPPAPPPPSSDPRDAC	This study
Goat	Human Scl	R&D Systems, AF3360
Goat and Rabbit	Human Sox2	Santa Cruz Biotechnology, sc-17320 and Bylund et al., 2003

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Chapter 3

Characterization of Pitx2-expressing cells in the spinal cord and hindbrain[1]

Introduction

Early in development, the homogeneous neuroepithelium becomes subdivided into discrete longitudinal, dorsal-ventral, and anterior-posterior domains. The central nervous system (CNS) initially forms by differentiation of the rostral portion of the neural tube into three vesicles: the prosencephalon, the mesencephalon, and the rhombencephalon which give rise to the forebrain, midbrain, and hindbrain, respectively. These structures are further divided into increasingly differentiated regions that will give rise to specific brain structures. The hindbrain is the part of the CNS that is responsible for coordination of motor processes and regulation of autonomic processes such as respiration, heart rate, blood pressure, and arousal (Linzey, 2001). It is located between the spinal cord and midbrain and in primitive vertebrates is the largest of the three brain regions. The cerebellum is the largest, most complex, and most well characterized hindbrain derivative and is responsible for integration of motor and sensory systems in the control of movement as well as in cognitive and emotional processes such as language, attention, fear, and reward responses (Kandel et al., 2000; Glickstein et al., 2009; Strick et al., 2009).

The developing hindbrain is subdivided into seven rhombomeres along the anterior-posterior axis which are distinguished by boundaries of gene expression, patterns of cell differentiation, and morphology (Mucchielli et al., 1996; Kiecker and Lumsden, 2005). While initially defined by broad expression of particular transcription factors, cells within each progenitor domain become increasingly specified, with groups of postmitotic neurons expressing unique transcription factors that direct formation of specific hindbrain nuclei with distinct functions (Mucchielli et al., 1996; Aroca and Puelles, 2005; Kiecker and Lumsden, 2005).

Pitx2 (also referred to as *ARP1, Brx1, Otlx2, PTX2, RGS, RIEG, RIEG1, solurshin*) is a paired-like homeobox gene that plays important roles in the development of a number of tissues. It is involved in the regulation of left-right asymmetry in the developing embryo (Schweickert et al., 2000; Campione et al., 2001) and is required for the development of numerous organs, such as pituitary, eyes, teeth, palate, heart, and limbs (Gage et al., 1999). In the murine central nervous system, expression of *Pitx2* is detected as early as E9.5 in the basal plate near the mesencephalic flexure and becomes more prominent by E10.5 from the mesencephalon to the mammillary region including the nascent zona limitans (Mucchielli et al., 1996). By E12.4-14.5 *Pitx2* is strongly expressed in the hypothalamus and rostral mesencephalon (superior colliculus). There is also a small group of cells in the basal plate of rhombomere 1 (r1) and a column of cells within the ventral spinal cord, dorsal to the motor columns (Mucchielli et al., 1996; Martin et al., 2002). In mice, complete loss-of-function mutations in *Pitx2* cause embryonic lethality by E15 with severe defects in heart, craniofacial structures, and the pituitary gland (Gage et al., 1999).

Mutations in *PITX2* are a cause of Axenfeld-Rieger Syndrome (ARS) in humans (Semina et al., 1996; Hjalt and Semina, 2005; Tumer and Bach-Holm, 2009). ARS is a rare autosomal-dominant disorder with high penetrance and variable expressivity. It is characterized by anomalies in the anterior chamber of the eye that cause glaucoma, dental hypoplasia, craniofacial dysmorphism, and umbilical abnormalities (Hjalt and Semina, 2005; Tumer and Bach-Holm, 2009). Some affected individuals also show limb malformations, growth hormone deficiency, and a variety of gastrointestinal defects such as Meckel's diverticulum and colonic atresia (Semina et al., 1996). These phenotypic defects are consistent with *Pitx2* expression in the periocular mesenchyme, oral epithelium, pituitary, and limb mesenchyme, although there is no direct correlation between particular mutations in the *PITX2* gene and the severity of the phenotype observed (Tumer and Bach-Holm, 2009).

The *PITX2* gene and its expression are highly conserved across species (Mucchielli et al., 1996; Amand et al., 1998; Lindberg et al., 1998). The *Pitx2* gene is about 20 kb long, with six exons ranging in size from 56 bp to 1.2 kb that encode multiple transcripts through alternative splicing and promoter usage (Cox et al., 2002). Conserved domains include the DNA-binding homeodomain and the C-terminal *aristaless* domain in exon 6 that mediate protein-protein interactions (Cox et al., 2002). Three major isoforms of *PITX2* have been identified in species as diverse as human, mouse, chick, zebrafish, and frog that share common C-termini and homoeodomain regions but have diverse N-termini. *PITX2*a and *PITX2*b are generated by differential mRNA splicing (Fig. 3.9 Supplementary Figure S1). *PITX2*a is 271 amino acids long and contains exons 1, 2, 5, and 6. *PITX2*b consists of exons 1-3, 5, and 6 which encodes

a protein of 317 amino acids. *PITX2*c, encoding a protein of 324 amino acids, uses an alternative promoter located in intron 3 and consists of exons 4-6 for a unique N-terminus sequence (Schweickert et al., 2000). A *PITX2*d isoform has been identified in humans that uses the alternative promoter in intron 3 to encode a 205 amino acid protein consisting of a truncated homeodomain with the complete C-terminal tail (Cox et al., 2002). PITX2d does not bind DNA and does not contain a functional homeodomain, but appears to negatively regulate the activity of *PITX2*a and *PITX2*c. More recently, additional variants of *Pitx2b* and *Pitx2c* have been identified in humans and in mouse with as yet unidentified distinct roles (Lamba et al., 2008). The three major *PITX2* isoforms are differentially expressed in various tissues and may regulate target gene expression through heterodimerization, providing a mechanism for fine-tuning gene expression during development. Careful control of functional levels of PITX2 protein is important as dosage-sensitive effects have been reported, with both increased and decreased levels causing defects in proliferation and development of various organs (Gage et al., 1999; Huang et al., 2010).

Pitx2 mRNA can be detected in mouse forebrain by E10.5 in the mantle zone of the ventral diencephalon and in the mesencephalon where differentiating neurons are located (Mucchielli et al., 1996; Martin et al., 2002). By E12.5, *Pitx2* expression becomes concentrated in several regions, including the zona limitans intrathalamica, retromammillary region, and mammillary region in the diencephalon, the pretectum, and broadly across the mesencephalon in addition to strong expression in the anterior pituitary anlagen, Rathke's pouch. Between E10.5 and E12.5, a period of robust neurogenesis in the mouse, the population of $PITX2^{+}$ cells in both diencephalon and

mesencephalon expand markedly and coexpress the neuronal marker *Tuj1* (Martin et al., 2002). All of these areas are morphologically normal in *Pitx2* mutant mice, as are the vesicles, flexures, and prosomeres, indicating that *Pitx2* does not appear to be involved in initial patterning of the neural tube or in regulation of neurogenesis. No differences in proliferation or apoptosis in *Pitx2* mutant brain have been reported (Martin et al., 2004; Skidmore et al., 2008).

Pitx2 activates transcription of glutamic acid decarboxylase (Gad67) in neural progenitor cells, an important enzyme in GABA synthesis, and the *Pitx2* homolog in C. elegans, *unc-30*, is essential for differentiation of all GABAergic neurons (Westmoreland et al., 2001; Sanchez et al., 2006). Consistent with these findings, in the dorsal mesencephalon, *Pitx2* labels a subset of GABA-expressing neurons from E10.5 through E14.5 in an area that will give rise to the superior colliculus (Martin et al., 2002). However, in the ventral diencephalon, *Pitx2* and GABA are not uniformly coexpressed at E10.5. By E12.5, there is coexpression of *Pitx2* and GABA in the zona incerta/retromammillary region, whereas in the zona limitans intrathalamica (zli), *Pitx2* and GABA are expressed in adjacent regions suggesting that zli cells are not GABAergic (Martin et al., 2002). At E14.5, *Pitx2* and GABA continue to be coexpressed in the zona incerta/retromammillary region, but populations in the subthalamic nucleus, supramammillary nucleus, medial mammillary nucleus, and posterior hypothalamus are GABA-negative. Subthalamic nucleus neurons coexpress *Pitx2* and calretinin and this calretinin expression is lost in *Pitx2* mutants (Martin et al., 2004).

In the superior colliculus of the dorsal midbrain at E14.5, most $Pitx2^{+}$ cells are GABAergic and localized to the stratum griseum intermedium layer (Waite et al., 2010).

However, although many of the intermediate zone GABAergic cells expressed the GABAergic differentiation markers Gata2 or Lhx1/5, the Pitx2^{+} cells do not, indicating that these cells form a unique GABAergic population within the superior colliculus (Waite et al., 2010). Two discrete ventral populations of *Pitx2*-expressing cells are also evident in the mesencephalon by E14.5, one in the red nucleus and a second in a more ventro-medial location. In the red nucleus and ventro-medial groups, Pitx2^{+} cells coexpress vesicular glutamate transporter type 2 (vGluT2) and the transcription factor Brn3a, marking them as glutamatergic, although complete transcription factor profiles differ between the two groups (Waite et al., 2010). These studies highlight diversity in the phenotypes of *Pitx2*-expressing neurons in the developing mouse brain.

By midgestation, Pitx2^{+} cells have undergone substantial migration and are more dispersed. In particular, the appearance of Pitx2^{+} cells in subthalamic and mammillary nuclei result from migration of these neurons and their organization into discrete areas. In the E14.5 mesencephalon of *Pitx2* null mice, in which an insertion into exon 4 results in a truncated, nonfunctional protein, there are substantial differences in the distribution of *Pitx2* mRNA compared to controls. *Pitx2* mRNA is observed near the intermediate and subventricular zone in the developing superior colliculus in the mutant animals, whereas in controls, it is located near the pial surface (Martin et al., 2004). Retrograde tracing indicates that *Pitx2* cells fail to develop normal projections and are compromised in their ability to migrate towards the pial surface in the mesencephalon. Similar defects are seen in the diencephalon of *Pitx2* mutant mice, where *Pitx2* mRNA expression is lost in the subthalamic nucleus but expanded in intermediate and subventricular zones of the ventrolateral thalamic area where retrograde tracing indicates that projections from the

subthalamic nucleus to the tegmentum are absent (Martin et al., 2004). These defects in migration were confirmed by cre-mediated fate mapping in which the migration of *Pitx2*-expressing neurons, marked by perdurant expression of ß–gal and birth-dated using BrdU, was shown to be disrupted or arrested shortly after E12.5 (Skidmore et al., 2008). In contrast, normal patterns of *Pitx2* mRNA expression were observed in the zona limitans and mammillary regions, as well as in rhombomere 1 of the hindbrain in mutant animals. This suggests that there are pleiotropic and regional differences in the function of *PITX2* during CNS development.

In more caudal regions of the CNS, *Pitx2* is expressed in subsets of cells in the hindbrain and spinal cord. The spinal cord PITX2 population was recently characterized after identification using a microarray screen to detect genes selectively enriched in ventral spinal cord of P8 mice (Zagoraiou et al., 2009). As previously described (Mucchielli et al., 1996), *Pitx2* positive cells are present in longitudinal clusters of cells in the intermediate zone of the spinal cord beginning around E10.5 at cervical and thoracic levels and E11.5 at lumbar levels and continuing postnatally (Zagoraiou et al., 2009). Genetic lineage tracing using a Dbx1::nlsLacZ reporter line (Pierani et al., 2001), which marks neurons that originate in the $Dbx1^+$ p0 progenitor domain, identified $PITX2^+$ cells as a subset of V0 interneurons (Zagoraiou et al., 2009). *Pitx2* was expressed by 5% of all $LacZ^+$ neurons at E12.5, marking them as a small subset of this population (Zagoraiou et al., 2009).

V0 interneurons can be subdivided into two populations, those that express *Evx1/2* (V0V) and those that do not (V0D) (Lanuza et al., 2004). $PITX2^+$ neurons coexpress *Evx1/2* transiently soon after differentiation, identifying them as a subset of the

V0V population (Zagoraiou et al., 2009). Furthermore, within the PITX2$^+$ subset of neurons, two phenotypic classes exist based on neurotransmitter expression. Some of these neurons coexpress choline acetyl transferase (ChAT, a critical synthetic enzyme for acetylcholine production) and vesicular acetylchoine transporter (vAChT), marking them as cholinergic, whereas other PITX2$^+$ cells express vGluT2, marking them as glutamatergic. No cells in the P8 spinal cord express both markers, suggesting that PITX2$^+$ cells represent distinct subpopulations designated V0C and V0G (Zagoraiou et al., 2009). Some PITX2$^+$ V0C neurons may also coexpress nitric oxide, which can act as a neurotransmitter (Enjin et al., 2010). Examination of the distribution of V0C and V0G neurons along the anterior-posterior axis of the P8 lumbar spinal cord shows differential distribution of the two phenotypic subsets. PITX2$^+$ V0C neurons predominate at more rostral lumbar levels, whereas at more caudal levels, the majority of PITX2$^+$ neurons are V0G.

Connectivity of neurons can be traced using conditional reporters that label neuronal processes. PITX2-expressing neurons were marked by crossing a *Pitx2::Cre* mouse line with reporter strains that can conditionally express fluorescent protein (GFP/YFP) in neurons (Zagoraiou et al., 2009). Using this system, cre recombinase is expressed exclusively in PITX2$^+$ neurons, turning on YFP expression in the cell bodies and processes of these neurons and allowing examination of connectivity (Zagoraiou et al., 2009). The cell bodies and proximal dendrites of motor neurons in the ventral horn of the spinal cord are covered with large vAchT$^+$ boutons, known as C boutons, expressing YFP. Cholinergic inputs modulate neuronal output and behavior in many regions of the CNS and C boutons are the most prominent source of cholinergic input to motor neurons

in the spinal cord (Conradi and Skoglund, 1969; Li et al., 1995). Studies showing that activating the C bouton postsynaptic muscarinic receptors on motor neurons increase motor neuron firing by reducing after-spike hyperpolarization and, conversely, that blocking muscarinic receptors decreases motor neuron output (Miles et al., 2004) led to the hypothesis that C bouton synapses modulate motor output. Muscarinic receptors are positioned opposite the YFP-expressing terminals observed on the motor neurons, indicating that Pitx2^{+} V0C neurons are the source of C boutons (Zagoraiou et al., 2009). Further tracing of connections indicates that Pitx2^{+} V0C neurons project ipsilaterally and receive excitatory inputs from glutamatergic interneurons, inhibitory inputs from GABAergic interneurons, and brainstem serotonergic inputs. Other ventral interneuron classes have been implicated in modulation of motor neuron output, such as V2a interneurons and Renshaw cells (Alvarez and Fyffe, 2007; Crone et al., 2008), but these do not appear to be contacted by PITX2^{+} V0C neurons. PITX2^{+} V0G neurons have not been extensively examined, but appear to contact other uncharacterized spinal interneurons in local circuits within the spinal cord rather than sending synapses to motor neurons as do V0C neurons.

Physiological characteristics of V0C neurons have also been studied in spinal cord explant preparations. Results from these studies indicate that firing of PITX2^{+} neurons is linked in phase to ipsilateral motor neurons located in the same segmental position, but that they lack intrinsic rhythmogenic properties. Phasic firing of V0C neurons is driven by locally active excitatory interneuron circuits. The *in vivo* contribution of V0C neurons to motor activity was studied in transgenic mice in which ChAT expression was selectively eliminated from Pitx2^{+} neurons using *Dbx1::Cre;ChAT*$^{fl/fl}$ mice (Zagoraiou et

al., 2009). These mice were viable through adulthood and had normal numbers of vAChT^{+} C boutons aligned with muscarinic receptors although no acetylcholine was present at these synapses. Behavioral assays of walking and swimming locomotion, which require different degrees of hindlimb muscle activation, were used to examine the effects of loss of PITX2^{+} V0C neurons. The pattern of locomotion during walking and during swimming was similar between control and Dbx1::Cre;ChAT$^{fl/fl}$ mice. However, in *Dbx1::Cre;ChAT$^{fl/fl}$* mice, the expected increased hindlimb muscle activation during swimming was significantly reduced compared to controls. These results suggest that PITX2^{+} V0C neurons form an important component in task-dependent modulation of motor neuron activity in hindlimb muscles (Zagoraiou et al., 2009). Importantly, this study also showed that the p0 progenitor domain gives rise to a larger variety of interneuron subtypes than previously known, implying there are many more distinct ventral interneuron subclasses than identified to date based on physiological or anatomical classification.

Most V0 interneurons are inhibitory, either GABAergic or glycinergic. In contrast, PITX2^{+} V0 neurons are excitatory, with either acetylcholine or glutamate as neurotransmitters. Inhibitory V0 interneurons project their axons contralaterally (Pierani et al., 2001), whereas excitatory PITX2^{+} V0 neurons send axons ipsilaterally. This extent of diversity from a single cardinal progenitor domain suggests there must be complex mechanisms of progressive specification within this population, likely involving cell–intrinsic and –extrinsic influences as is the case in motor neuron columnar and pool identity specification (Dasen et al., 2005; Dasen et al., 2008; Rousso et al., 2008).

In contrast to recent advances in defining the *Pitx2*-expressing cells in spinal cord, the characteristics of cells that express *Pitx2* in r1 of the developing hindbrain have yet to be elucidated. *Pitx2* expression is seen in a subset of neurons in ventral r1 by E12.5. Earlier studies suggested that these cells are GABAergic (Martin et al., 2002) but no further characterization was performed. The purpose of this study was to describe the characteristics of *Pitx2*-expressing neurons with respect to developmental time course, transcription factor complement, neurotransmitter phenotype, and functional connectivity within the hindbrain.

Materials and Methods

Animal and tissue preparation

Timed pregnancies were established with the morning of plug identification designated as E0.5. *Pitx2*$^{cre/+}$, *Pitx2*$^{cre/+}$*;N-lacZ/N-lacZ, and Pitx2*$^{cre/null}$ mice were generated as previously described (Skidmore et al., 2008). Wild-type mice were on a C57BL/6J background (JAX 000664). Litters of embryos were dissected into PBS from pregnant females following cervical dislocation and hysterectomy. Genotyping was carried out on an amniotic sac, tail, or limb from each embryo. Embryos were fixed and processed for antibody staining or *in situ* hybridization histochemistry as previously described (Novitch et al., 2001; Rousso et al., 2008; Skidmore et al., 2008). Briefly, embryos were fixed in 4% formaldehyde at 4°C for 30 min to 4 h at depending on age. For frozen sections, embryos were cryoprotected overnight in 30% sucrose, flash frozen in O.C.T. embedding compound, and stored at -80°C until sectioning at 12-15 μm. For paraffin sections, tissues were embedded in paraffin and sectioned at 7 μm thickness. For vibratome

sections, embryos were post-fixed in 4% formaldehyde overnight at 4°C, craniofacial tissues removed and brains embedded in 4% low melt GenePure agarose (BioExpress, Kaysville, UT), and sectioned at 150 μm. All procedures were approved by the University Committee on Use and Care for Animals at the University of Michigan.

β-galactosidase assay

Embryos were dissected and fixed in 4% formaldehyde for 20–30 min and then washed in PBS and X-gal wash Buffer, as previously described (Sclafani et al., 2006).

Immunofluorescence and in situ hybridization

Immunofluorescence on paraffin embedded tissues was done as previously described (Martin et al., 2002; Martin et al., 2004). Immunofluorescence on frozen sections was done as previously described (Novitch et al., 2001; Rousso et al., 2008). Antibodies used were rabbit anti-PITX2 at 1:400 (generously provided by Dr. Tord Hjalt, Lund University, Sweden), rabbit anti-PITX2 at 1:8000 (generously provided by Dr. Thomas Jessell, Columbia University), rabbit anti-PITX2 at 1:4000 (Capra Science, Ängelholm, Sweden), rat anti-β-galactosidase at 1:1000 (generously provided by Dr. Tom Glaser, University of Michigan), rabbit anti-VGluT2 at 1:1000 (Millipore), rabbit anti-GABA at 1:1000 (Sigma), rabbit anti-5HT at 1:5000 (Sigma), goat anti-CHAT at 1:100 (Millipore), guinea pig anti-Bhlhb5 at 1:32000 (generously provided by Dr. Ben Novitch, University of California, Los Angeles), rabbit anti-LBX1 at 1:10000 (provided by Thomas Müller), guinea pig anti-LMX1B at 1:5000 (generously provided by Dr. Thomas Müller, Max-Delbrück Center of Molecular Medicine, Berlin), rabbit anti-SOX2 (Millipore) at 1:250, and the following mouse antibodies from Developmental Studies Hybridoma Bank at 1:100-1:150: anti-LHX1/5 (4F2), anti EVX1 (3A2), anti-EN1 (4G11), and anti-ISL1

(39.4D5). *In situ* hybridization on frozen and paraffin sections was done as previously described (Martin et al., 2002; Martin et al., 2004) using a cRNA probe for *Pitx2*.

Results

Pitx2 marks a subset of V0 interneurons in spinal cord

A small group of *Pitx2*$^{+}$ cells were initially described using whole mount *in situ* hybridization on E12.5 ventral spinal cord in a position dorsal to the location where motor neurons are generated (Mucchielli et al., 1996). We observed PITX2 immunoreactivity in a small group of cells in a ventral position within the E12.5 spinal cord (Fig. 3.1A-B). We sought to determine the specific identity of this population of neurons using double fluorescent antibody staining with antibodies against PITX2 and markers of known ventral interneuron subtypes (Jessell, 2000; Briscoe and Ericson, 2001). Distinct classes of spinal interneurons emerge from discrete progenitor domains stereotypic fashion during early neural development and can be identified by the transcription factors they express. dI6, V0, and V1 neurons are marked by expression of *Lbx1*, *Evx1/2*, and *En1* respectively. *Bhlhb5* is a marker of the dI6, V1, and V2 subgroups of spinal interneurons but is specifically excluded from V0 neuronal subtypes. We found that *Pitx2* is coexpressed with the V0 marker *Evx1/2* (Fig. 3.1F-H), although only a small subset of *Evx1/2*-expressing cells are also positive for *Pitx2*. There is no overlap with *Lbx1* or *En1/Bhlhb5*, markers for dI6 and V1 interneurons, respectively (Fig. 3.1C-E, I-N). These results indicate that *Pitx2* is expressed in a subset of V0 interneurons in the ventral spinal cord.

My results are in agreement with those of a recently published study that established the identity and function of *Pitx2*-expressing cells in the spinal cord (Zagoraiou et al., 2009). *Pitx2* marks a subset of V0 interneurons, representing about 5% of the total number of neurons that are generated from $DBX1^+$ p0 progenitors. These $PITX2^+$ interneurons are further subdivided into two groups on the basis of neurotransmitter phenotype: a cholinergic and a glutamatergic group (Zagoraiou et al., 2009). Although all $Pitx2^+$ neurons express *Evx1/2* at E12.5, at E14.5 *Evx1/2* expression is no longer evident in these cells and the *Pitx2* and *Evx1/2* populations are distinct. These results together with our observations identify *Pitx2* as marking two small populations of V0 interneurons: cholinergic ($V0_C$) and glutamatergic ($V0_D$).

Pitx2 is expressed in a discrete subset of hindbrain neurons in rhombomere 1

Pitx2 expression in the developing brain is most abundant in groups of postmitotic neurons in the telencephalon and diencephalon (Mucchielli et al., 1996; Lindberg et al., 1998; Martin et al., 2002). *Pitx2* is also found in the ventral hindbrain of the most rostral rhombomere, r1. A small area of $Pitx2^+$ cells can be found in the prospective r1 region as early as E9.5 (Fig. 3.2A) in parasagittal sections. Pitx2 mRNA and protein expand from E10.5 to E14.5, a period of rapid neurogenesis in mouse. Multiple labeling methods detect *Pitx2* expression during this time period. *Pitx2* mRNA is readily detected in r1 at E12.5 in a distinct set of cells in the ventral area (Fig. 3.2B). X-gal staining detects *Pitx2*-driven ß-galactosidase activity in r1 at E14.5 in $Pitx2^{cre/+}$*;N-lacZ/N-lacZ* mouse embryos (Fig. 3.2C), previously generated for lineage tracing studies (Skidmore et al., 2008).

Pitx2 mRNA is present by *in situ* hybridization in transverse sections through E12.5 (Fig. 3.2D-E) and E14.5 (Fig. 3.2F) although it is much less abundant in r1 than in the opposing hypothalamus. In E14.5 sagittal sections, *Pitx2* label can be clearly detected in a distinct group of cells in the ventral area of r1 (Fig. 3.2G-I). This pattern of expression suggests that *Pitx2* may mark a specific nucleus or functional subgroup in ventral r1.

Pitx2-expressing neurons in rhombomere 1 are GABAergic

Pitx2 expressing cells are known to have a variety of neurotransmitter phenotypes in different regions of expression. In the spinal cord, *Pitx2*$^+$ cells have one of two neurotransmitter phenotypes: cholinergic or glutamatergic (Zagoraiou et al., 2009). In both the diencephalon and mesencephalon, many areas of GABAergic PITX2$^+$ cells are found, including in the superior colliculus, zona incerta/retromammillary region (Martin et al., 2002). However, PITX2$^+$ cells in the zona limitans intrathalamica, subthalamic nucleus, supramammillary and medial mammillary nuclei, posterior subthalamic region, and ventral pretectal area do not express GABA (Martin et al., 2002; Skidmore et al., 2008; Waite et al., 2010). Recent detailed analyses of *Pitx2* expression in midbrain determined that ventral medial and red nucleus neurons in the ventral midbrain are glutamatergic, in contrast to those in the superior colliculus (Waite et al., 2010). Earlier work suggested that PITX2$^+$ cells in r1 have a GABAergic phenotype (Martin et al., 2002). It appears that the r1 Pitx2 population may indeed be GABAergic (Fig. 3.3A-I), although additional confirmation with co-staining using additional markers of GABAergic neurons is needed. In addition, a wide field of VGluT2 expression is

observed in r1 surrounding *Pitx2*-expressing cells (Fig. 3.3J-O). The pattern of VGluT2 expression may represent tracts passing through the hindbrain or glutamatergic inputs to *Pitx2*-expressing cells in r1.

Two of the most well characterized hindbrain nuclei are the serotonergic raphe nuclei and the noradregeneric locus coeruleus (Zervas et al., 2005; Aroca et al., 2006). We used double immunofluorescence with antibodies against PITX2 and 5-hydroxytryptamine (5-HT) to investigate the location of Pitx2 expression with respect to that of serotonin. There was no overlap between *Pitx2* expression and 5-HT, indicating that the $Pitx2^{+}$ neurons are not members of the raphe nuclei (Fig. 3.4A-F). Serotonin- and *Pitx2*-expressing cells intermingle at the ventromedial limits of *Pitx2* expression, but are distinguished by morphological differences between the cell types as well as by lack of co-expression. Locus coeruleus neurons arise from the dorsal portion of r1 and subsequently migrate to more ventral locations (Aroca et al., 2006). Locus coeruleus neurons can be distinguished based on expression of the transcription factors Phox2a and Phox2b and the noradrenergic enzyme dopamine-ß-hydroxylase (DBH). Expression of Phox2a, Phox2b and DBH with respect to *Pitx2* have yet to be examined, but could help refine the precise organization of nuclei in ventral r1. Based on the apparent GABAergic neurotransmitter expression and dorsal derivation of locus coeruleus neurons, co-expression seems unlikely.

Several populations of cholinergic neurons in the hindbrain have been identified, including the cranial motor nuclei and the parabrachial complex (Mizukawa et al., 1986). Interestingly, a subset of *Pitx2*-expressing cells in spinal cord are cholinergic neurons (Zagoraiou et al., 2009). We assessed the relationship between *Pitx2* expression and

cholinergic populations in hindbrain by immunofluorescence using an antibody against choline acetyl transferase (ChAT), an enzyme required for synthesis of acetylcholine and comparing its expression to that of *Pitx2* on near adjacent sections. *Pitx2* and ChAT expression were not observed in the same areas of ventral r1 (Fig. 3.4G-H). A few $ChAT^{+}$ cells were observed caudal to the group of $PITX2^{+}$ neurons but appear to mark distinct neuronal groups.

Pitx2 is not expressed in progenitor cells in ventral r1

In most areas of the brain, *Pitx2* is expressed in post-mitotic cells but not in neuronal progenitors. To determine whether this is also true of *Pitx2* expression in r1, we used immunofluorescence with antibodies against PITX2 and the neural progenitor marker SOX2 (Pevny and Placzek, 2005). Consistent with *Pitx2* expression in other brain areas, we observed no overlap between *Pitx2* and *Sox2* expression (Fig 3.5). Interestingly, $Sox2^{+}$ cells surround $Pitx2^{+}$ cells at E12.5, suggesting that this area is undergoing active neurogenesis at this stage of development and that some neurons may maintain *Sox2* expression at least transiently after differentiation.

$Pitx2^{+}$ neurons show colocalization with neurons expressing global r1 transcription factors

Fgf8 expression in the isthmic organizer is the primary signal regulating establishment of the midbrain/hindbrain boundary and directing expression of genes critical for midbrain and hindbrain development. The engrailed homeobox genes, *En1/2*, are induced by Fgf8 in a broad region encompassing the midbrain and anterior hindbrain

and are critical for midbrain and r1/cerebellum development (Zervas et al., 2005; Sgaier et al., 2007). Some cells positive for En1/2 reactivity in ventral hindbrain had been observed but not well characterized, so we examined possible coexpression with *Pitx2*. At E12.5 there is substantial coexpression of *Pitx2* and *En1/2* in ventral r1 although some *En1/2*$^+$ and a few *Pitx2*$^+$ singly labeled cells are also noted (Fig. 3.6A-F). It appears that the vast majority of *Pitx2*$^+$ cells in ventral r1 are *En1/2*$^+$ at E12.5.

LIM-homeodomain proteins are transcription factors expressed by many neuronal subtypes throughout the developing central nervous system (Shirasaki and Pfaff, 2002). Lhx1/5 are expressed widely throughout r1 and the developing cerebellum and are required for normal cerebellar development from dorsal r1 (Morales and Hatten, 2006; Zhao et al., 2007). We sought to determine using double immunofluorescence whether PITX2$^+$ cells in ventral r1 also express *Lhx1/5*. We found that at E14.5 virtually all of the *Pitx2*-expressing cells also express *Lhx1/5* (Fig 3.6G-L). The *Lhx1/5*$^+$ population of cells in ventral r1 represents a heterogeneous group of neurons and *Pitx2* expression may be used to define a distinct subpopulation within this group with potential functional significance.

Pitx2 does not mark dorsal raphe or cranial motor neurons

Lmx1b is a transcription factor involved in initiation and maintenance of the isthmic organizer and is thus important for midbrain and hindbrain patterning (Matsunaga et al., 2002; Zervas et al., 2005; Jacob et al., 2009; Prakash et al., 2009). It is also required for the differentiation of midbrain dopaminergic neurons and hindbrain serotonergic neurons (Guo et al., 2007; Jacob et al., 2009). *Isl1* is expressed by neurons

of the cranial nerves, including the oculomotor and trochlear nuclei that give rise to the third and fourth cranial nerves respectively (Prakash et al., 2009). Double immunofluorescent antibody staining showed that *Pitx2*-expressing cells do not belong to either of these populations (Fig. 3.7). Two groups of *Lmx1b*$^+$ cells are seen, one medial and one lateral, and the Pitx2$^+$ neurons are located between these two groups of cells (Fig. 3.7A-C). *Isl1* expression is localized to a more intermediate position along the dorsal-ventral axis of r1 rather than in the ventral region where *Pitx2*$^+$ neurons are located. These results are consistent with the lack of overlap between *Pitx2* and markers of serotonergic and cholinergic neurons in the hindbrain and suggest that *Pitx2* does not mark cranial motor neurons (see Fig. 3.4).

Discussion

In this study, we examined *Pitx2* expression in the developing spinal cord and rostral hindbrain. In spinal cord, Pitx2 marks a subset of V0 interneurons that derive from the Dbx1$^+$ p0 progenitor domain. Even though they represent a small subset of V0 interneurons, *Pitx2*-expressing cells exhibit two neurotransmitter phenotypes, cholinergic and glutamatergic. Recently the connectivity and function of the cholinergic V0$_C$ have been described in detail (Zagoraiou et al., 2009). These neurons provide cholinergic input to motor neurons in the form of C boutons and are implicated in local spinal locomotor circuits that modulate the amplitude of motor neuron firing in a task-dependent manner (Zagoraiou et al., 2009). Selective elimination of ChAT from V0C neurons does not disrupt the formation or organization of C bouton synapses on motor neurons (Zagoraiou et al., 2009) but the equivalent experiment to remove glutamate from the V0G

subpopulation has not been reported. Conditional removal of glutamate from the V0G subpopulation would produce potentially different results, since in contrast to acetylcholine, glutamate has been shown to influence axonal guidance and synapse formation (Ruediger and Bolz, 2007). The function and connectivity of the glutamatergic $Pitx2^{+}$ neurons, the $V0_G$ population, have yet to be determined.

An important addition to existing data on *Pitx2*-expressing neurons in spinal cord would be loss-of-function studies to determine the fate of $Pitx2^{+}$ V0 interneurons in the absence of *Pitx2*. The effects of complete elimination of *Pitx2* from V0 neurons could be investigated through examination of spinal neuron formation in embryos obtained from the crossing of mice containing a conditional *Pitx2* deletion allele (Skidmore et al., 2008) with mice expressing Cre recombinase under control of the p0 marker *Dbx1* (Pierani et al., 2001). The availability of a $Pitx2^{LacZ}$ allele along with the V0-*Pitx2* conditional null would facilitate investigation of potential fate switches by permanently marking presumptive $PITX2^{+}$ cells and allowing determination of their fate in the absence of *Pitx2* (Skidmore et al., 2008).

The finding that *Pitx2* marks two additional subgroups of V0 interneurons within the spinal cord highlights the probable existence of many more neuronal types whose identity is important for understanding the circuits that control the complex patterns of locomotion. The identification of *Pitx2* as a marker of two unique classes of interneurons is also an important contribution to unraveling this diversity and promoting a more complete understanding of local circuits that produce and modulate mammalian locomotion.

Pitx2 appears to mark a distinct set of neurons in ventral r1 in the developing hindbrain (Fig. 3.8). Much progress has been made in determining the cellular origins and interactions that give rise to the cerebellum, the most well-known and well-studied derivative of r1. In contrast, the definition and origins of the various nuclei and tracts of ventral r1 have received relatively little attention. Here we show that *Pitx2* marks a distinct set of neurons in the ventral intermediate zone in r1. Expression of *Pitx2* in r1 begins early, around E9.5, and continues at least through late gestation and early postnatal periods (data not shown). Many $Pitx2^{+}$ neurons in forebrain and midbrain appear to migrate and send projections between distant areas as shown by axon tracing and lineage marking studies (Martin et al., 2002; Martin et al., 2004; Skidmore et al., 2008; Waite et al., 2010). However the histological appearance of $Pitx2^{+}$ neurons in r1 is more suggestive of involvement in local circuitry. Future studies are needed to examine the interconnectivity of these cells and their particular function in r1.

Pitx2-expressing cells exhibit a variety of neurotransmitter phenotypes in different areas of the central nervous system, including GABAergic, glutamatergic, and cholinergic (Martin et al., 2002; Martin et al., 2004; Skidmore et al., 2008; Waite et al., 2010). In ventral r1, *Pitx2* appears to be expressed in GABAergic cells in agreement with earlier reports (Martin et al., 2002). No coexpression of *Pitx2* with serotonin or ChAT has been observed. $Pitx2^{+}$ cells are located within a field of glutamatergic processes, suggesting that they may receive or provide inhibitory modulation of signals in this area. $Pitx2^{+}$ cells also intermingle at their ventro-medial limit with serotonin-expressing cells presumed to mark the dorsal raphe nucleus. It is also possible that processes from $Pitx2^{+}$ cells might interact with those from neurons in the dorsal raphe in

ventral r1. Further confirmation of the neurotransmitter phenotype of *Pitx2* neurons must be obtained and axon tracing used to investigate the connections made by these neurons in relation to other neuronal groups in r1.

Throughout the central nervous system, different neuronal populations can be distinguished by the complement of transcription factors they express as well as by characteristics such as neurotransmitters expressed and characteristic projection patterns. We have provided a preliminary description of several of the transcription factors that are coexpressed with *Pitx2* in r1. The requirement for *En1/2* in development of the midbrain and cerebellum has been extensively studied (Aroca and Puelles, 2005; Zervas et al., 2005; Sgaier et al., 2007). *En1/2* is also expressed in a subset of ventral r1 neurons that have not been well characterized. Here we show that there is substantial overlap between *Pitx2* and *En1/2* expression in ventral r1 at E12.5. A more complete comparative developmental time course would establish the origins of $Pitx2^{+}$ cells, likely to derive from $Gbx2^{+}En1/2^{+}$ precursors (Aroca and Puelles, 2005; Zervas et al., 2005). In addition, we have shown that $Pitx2^{+}$ cells also coexpress the LIM-homeodomain transcription factors *Lhx1/5* that are widely expressed throughout r1. The observation that *Pitx2* expression does not coincide with that of *Lmx1b*, a marker of progenitor and serotonergic neurons in hindbrain, nor with *Isl1*, a marker of motor neurons in the oculomotor and trochlear nuclei, corroborates data acquired from the analysis of neurotransmitter phenotype by serotonin and ChAT immunoreactivity.

Much attention has been directed toward an understanding of the early and late molecular markers and the mechanisms of development of the cerebellum, the most prominent r1 derivative which has its origins in dorsal r1 (Herrup and Kuemerle, 1997;

Wang and Zoghbi, 2001; Melton et al., 2004; Aroca and Puelles, 2005; Hoshino et al., 2005; Zervas et al., 2005; Morales and Hatten, 2006; Zordan et al., 2008; Hashimoto, 2009; Jacob et al., 2009; Liu et al., 2010). Perhaps this is not surprising since the cerebellum is one of the first brain structures to differentiate and one of the last to mature, is estimated to contain as many as 80-85% of all human neurons, and is an important center for many processes critical for life (Herrup and Kuemerle, 1997; Wang and Zoghbi, 2001). In contrast, ventral r1 has received very little attention. Histologically it appears that *Pitx2* marks a distinct and coherent group of cells in poorly characterized regions of the hindbrain. Determination of the developmental origins and transcriptional cascades that regulate *Pitx2* expression will contribute to an overall understanding of how neuronal fates are specified in the developing hindbrain. This knowledge will extend understanding of r1 derivatives beyond cerebellum and may provide knowledge about markers of previously unidentified cell types in the hindbrain, similar to the discovery of two novel *Pitx2*-expressing neuronal classes in spinal cord (Zagoraiou et al., 2009).

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Chapter 3 Notes

[1]A revised version of Chapter 3 will be submitted for publication as Skaggs, K., Waite, M. R., Skidmore, J. M., Novitch, B. G. and Martin, D. M. GABAergic interneurons in rhombomere 1 exhibit unique transcriptional profiles.

Figure 3.1 *Pitx2* expression identifies a subset of V0 interneurons in spinal cord. Transverse sections through an E12.5 spinal cord were stained with antibodies against PITX2 and markers of various interneuron subtypes.

(A-B) PITX2 is expressed in a small subset of neurons in intermediate zone in the ventral spinal cord. (C-E) PITX2 is not coexpressed with LBX1, a marker of dI4-dI6 interneurons. (F-H) Coexpression of PITX2 and EVX1 identifies a subset of V0 interneurons. (I-K) PITX2 expression does not overlap with the V1 interneuron marker EN1/2. (L-M) BHLHB5 is expressed in subsets of dI6, V1, and V2 interneurons. BHLHB5 is not coexpressed with PITX2.

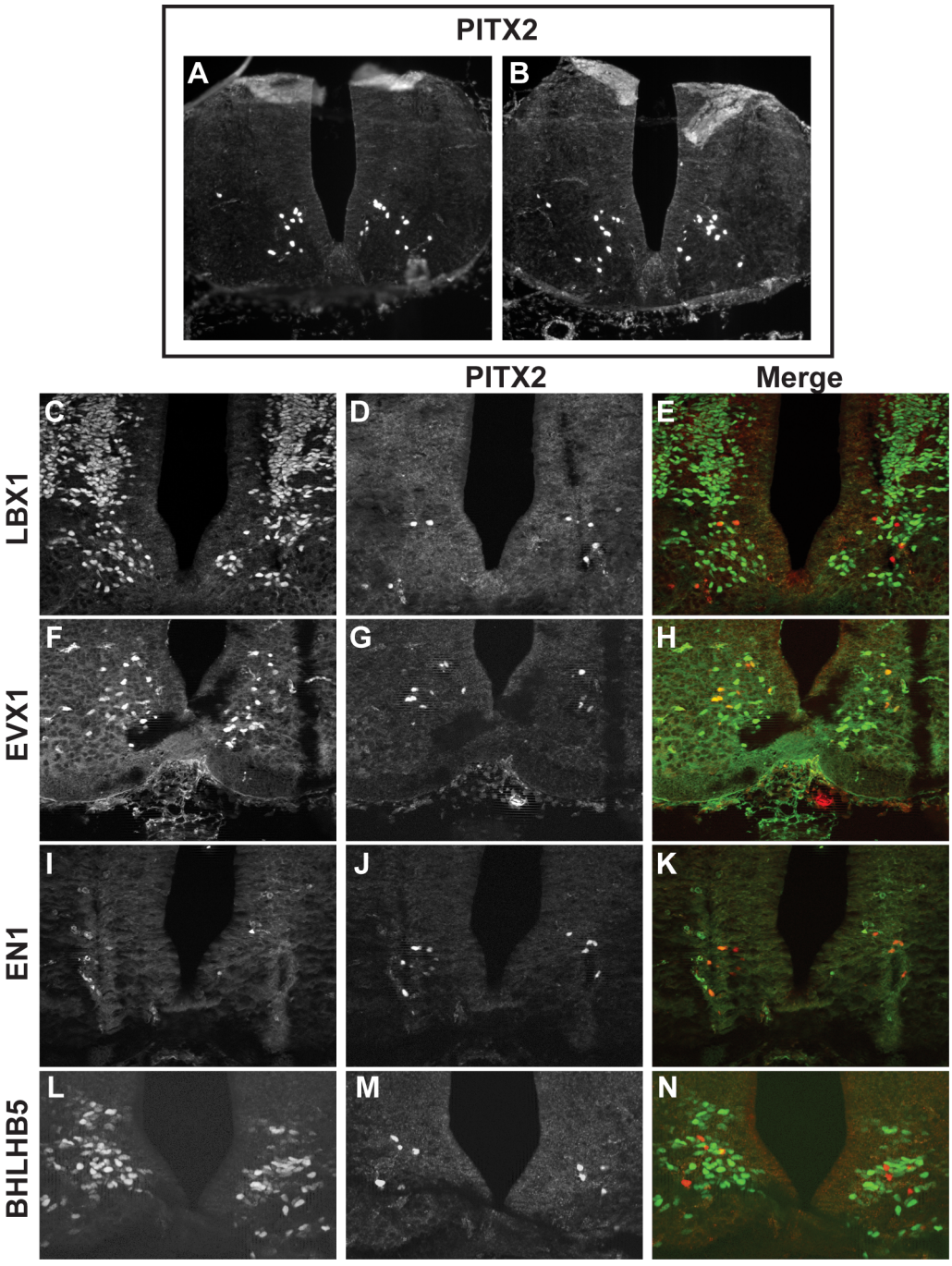

PITX2
A
B
PITX2
Merge
LBX1
EVX1
EN1
BHLHB5
C
D
E
F
G
H
I
J
K
L
M
N

Figure 3.2 *Pitx2* is expressed in distinct regions of ventral rhombomere 1 in the developing hindbrain. Various methods were used to identify PITX2+ cells from E9.5-E14.5. Circles in each image denote PITX2 expression in r1 of the hindbrain. In panels A-C and G-I, rostral is left and caudal is right. In panels D-E, ventral midbrain and ventral hindbrain are toward the center of each image and dorsal toward the outer top and bottom.

(A) Immunofluorescence with an antibody against PITX2 shows PITX2 is present very early in embryonic CNS development, at E9.5.

(B) *Pitx2* mRNA is expressed in r1 by E12.5. Panel from Mucchielli et al., 1996 and reversed to match rostral-caudal orientation of other panels.

(C) X-gal staining of a vibratome section from an E14.5 embryo shows *Pitx2*-driven ß-galactosidase expression in a broader area of ventral r1.

(D-F) *In situ* hybridization of transverse sections through hindbrain shows distinct groups of cells expressing *Pitx2* mRNA at E12.5 (D-E) and E14.5 (F). The level of *Pitx2* expression is much lower in r1 than in the adjacent hypothalamus.

(G-I) *In situ* hybridization of sagittal E14.5 embryonic brain shows *Pitx2* mRNA in a distinct but diffuse group of cells in ventral r1. Panels in H and I are enlargements of the $Pitx2^{+}$ cell region shown in G.

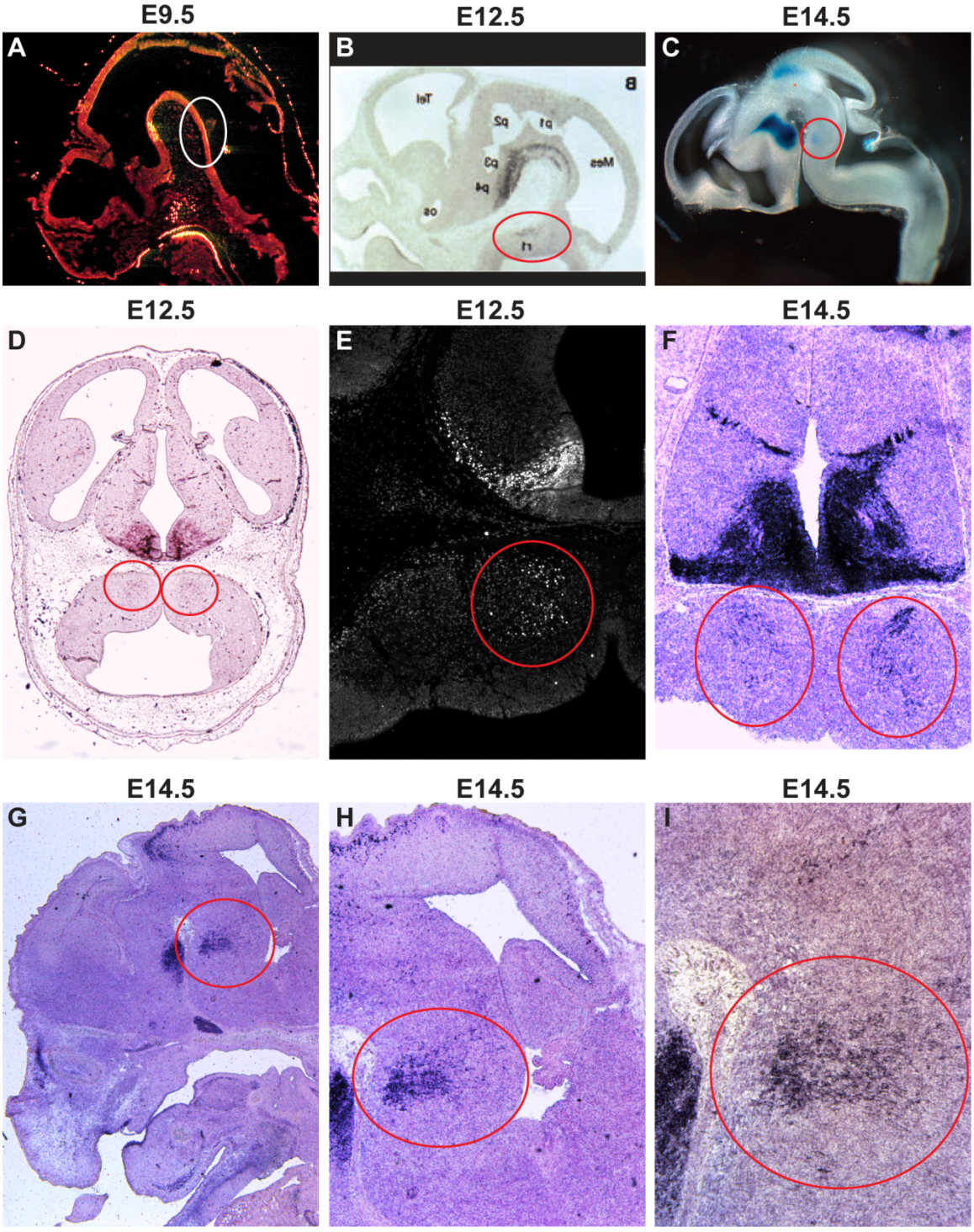

E9.5
E12.5
E14.5
A
B
C
E12.5
E12.5
E14.5
D
E
F
E14.5
E14.5
E14.5
G
H
I

Figure 3.3 *Pitx2* is expressed in a subset of GABAergic neurons in ventral r1.

Transverse sections at the level of r1 in E14.5 hindbrain were analyzed by double immunofluorescence for PITX2 and the indicated markers. In all panels, dorsal hindbrain is at the top and ventral hindbrain at the bottom.

(A-I) PITX2 expressing cells in r1 appear to coexpress GABA. D-F and G-I are enlargements of the boxed area in A-C.

(J-O) $PITX2^{+}$ cells are located within a field of VGluT2 reactivity but are VGluT2-negative. M-O are enlargements of boxed areas in panels J-L.

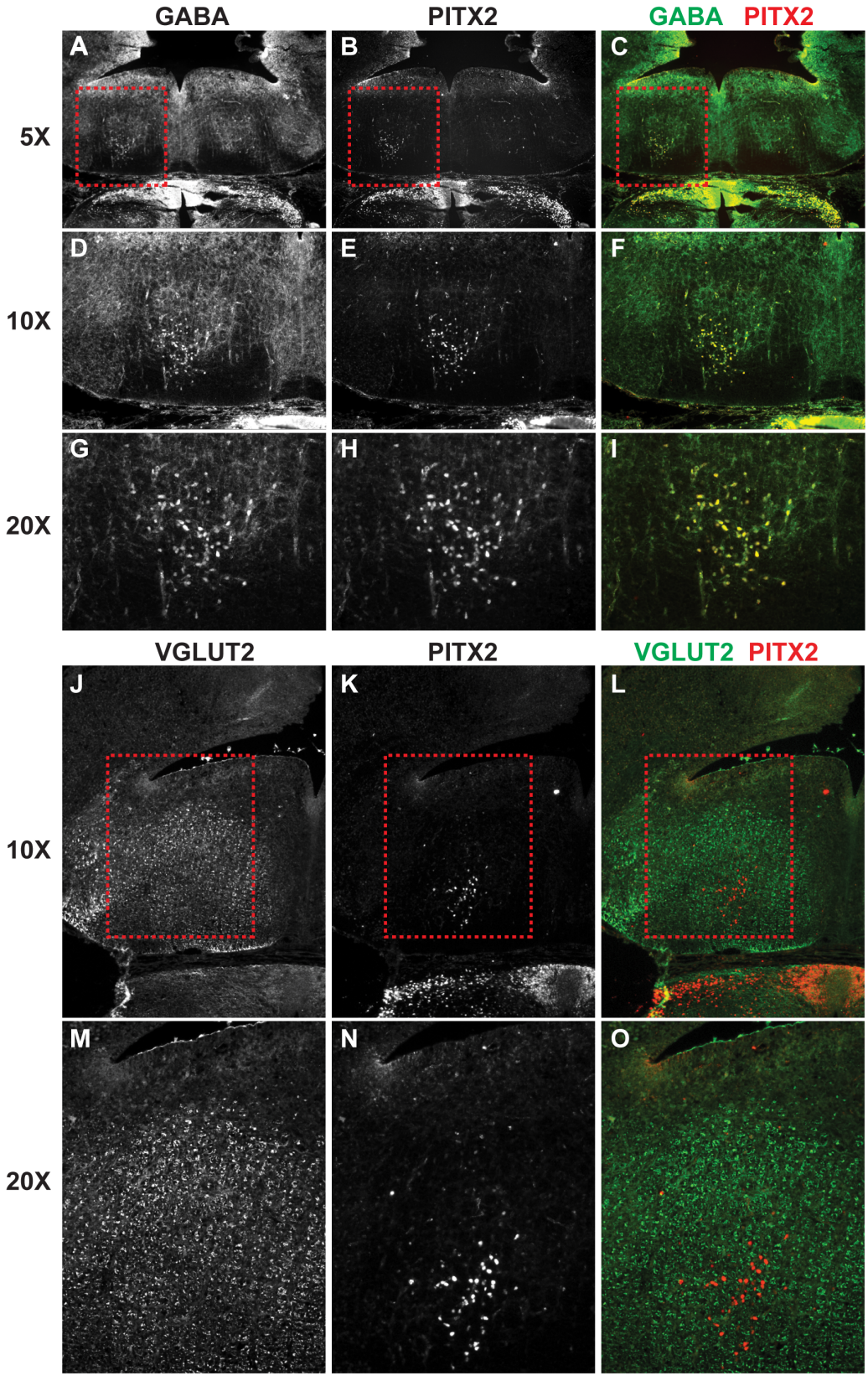
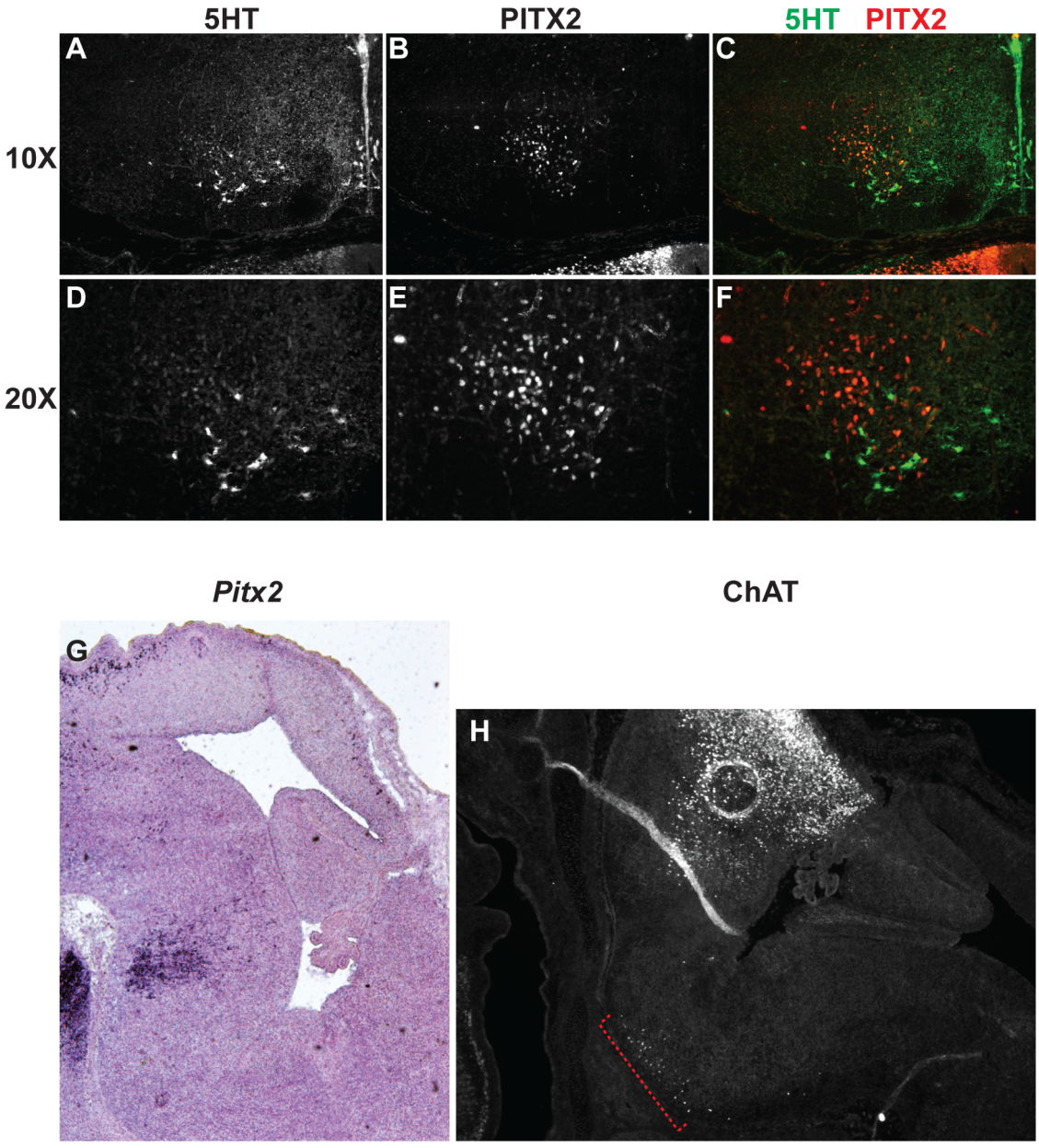

GABA
PITX2
GABA PITX2
A
B
C
5X
D
E
F
10X
G
H
I
20X
VGLUT2
PITX2
VGLUT2 PITX2
J
K
L
10X
M
N
O
20X

Figure 3.4 *Pitx2* expression does not mark serotonergic or cholinergic neurons in the rostral hindbrain. Double immunofluorescence with antibodies against 5-HT (serotonin) and PITX2 in transverse sections through r1 of an E14.5 hindbrain. In panels A-F, dorsal hindbrain is at the top and ventral hindbrain at the bottom of the image; in panels G-H, rostral is to the left and caudal to the right. D-F are higher magnifications of panels A-C.

(A-F) PITX2 does not mark serotonergic neurons, presumably of the dorsal raphe.

(G) *In situ* hybridization showing the location of *Pitx2* mRNA in r1 of a sagittal section of an E14.5 midbrain/hindbrain area.

(H) ChAT antibody staining in an adjacent section of E14.5 midbrain/hindbrain indicates that neurons in the area of *Pitx2* expression are not cholinergic. A small group of ChAT immunoreactive cells in ventral r1 caudal to the area of *Pitx2* expression are marked by the bracket.


5HT
PITX2
5HT PITX2
A
B
C
10X
D
E
F
20X
Pitx2
ChAT
G
H

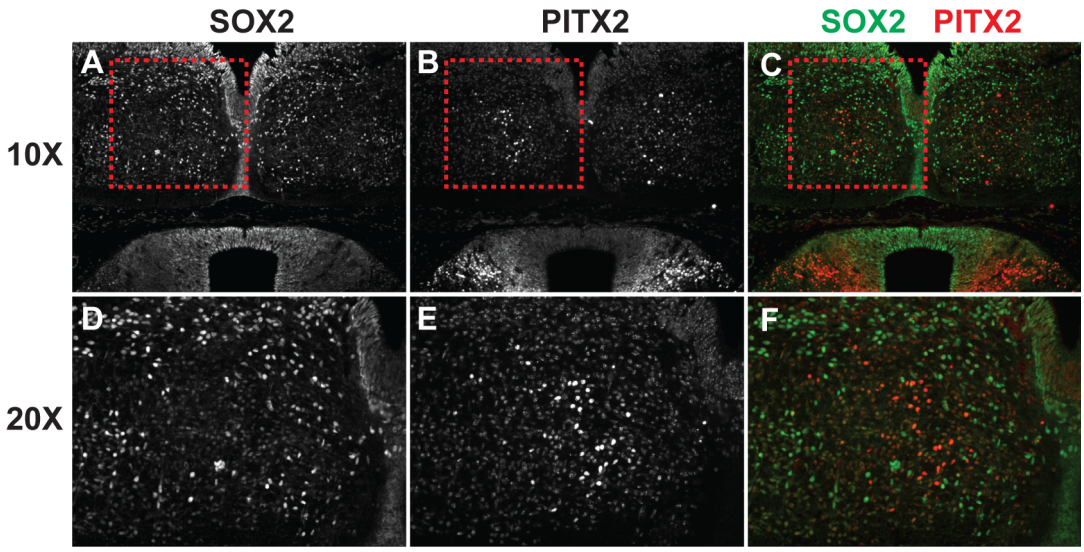
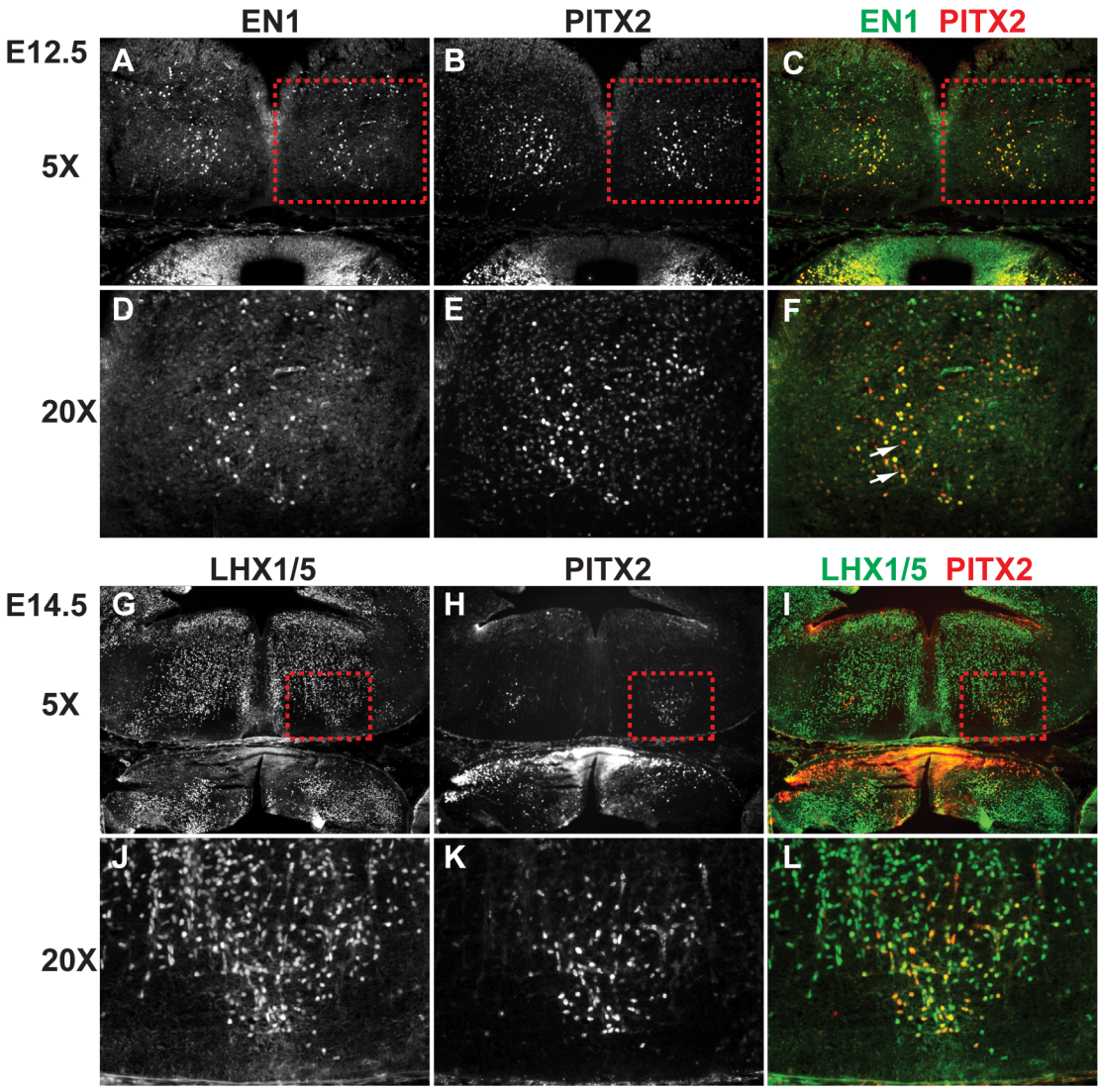


Figure 3.5 *Pitx2* is not expressed in progenitor cells in ventral r1.

In all panels, dorsal hindbrain is at the top and ventral hindbrain at the bottom of the image. (A-F) Double immunoflourescence for antibodies against the progenitor marker SOX2 and PITX2 indicates that PITX2 expression is confined to postmitotic neurons in r1.

Figure 3.6 *Pitx2* is coexpressed with the transcription factors EN1/2 and LHX1/5. Transverse sections through the r1 level of E12.5 hindbrain were analyzed by double immunofluorescence for PITX2 and EN1/2 or LHX1/5. In all panels, dorsal hindbrain is at the top and ventral hindbrain is at the bottom of the image. (A-F) The majority of cells are positive for both PITX2 and EN1/2. Arrows in F indicate $PITX2^{+}$ cells that do not coexpress EN1/2. (G-L) All PITX2 cells also coexpress LHX1/5.


EN1
PITX2
EN1 PITX2
E12.5
5X
A
B
C
20X
D
E
F
LHX1/5
PITX2
LHX1/5 PITX2
E14.5
5X
G
H
I
20X
J
K
L

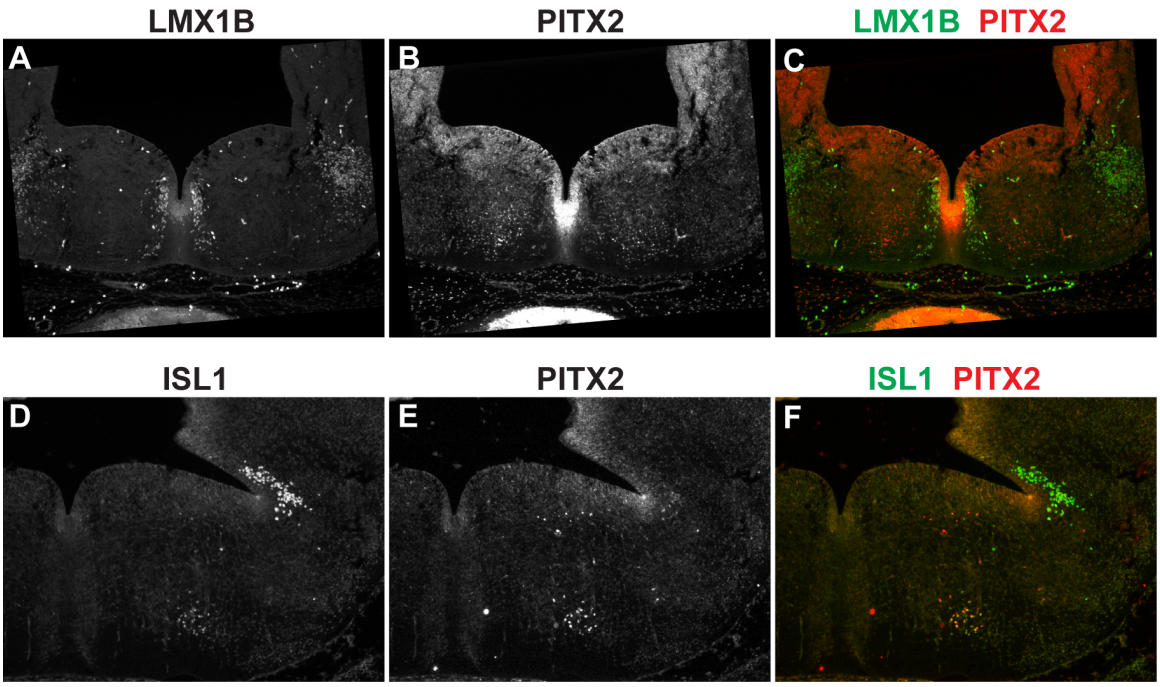


Figure 3.7 ***Pitx2*** **is not expressed in serotonergic neurons of the dorsal raphe or in cranial motor nerves.** Transverse sections at the r1 level of E14.5 hindbrain were analyzed by double immunofluorescence for PITX2 and the indicated markers. In all panels, dorsal hindbrain is at the top and ventral hindbrain at the bottom of the image. (A-C) Two populations of $LMX1B^{+}$ neurons are observed, in the medial and lateral ventral r1. $PITX2^{+}$ cells are located between the two $LMX1B^{+}$ groups. (D-F) There is no overlap between PITX2 expression and ISL1 which marks motor neurons of the trochlear nucleus. Faint staining in the ventral area of panel D is non-specific species cross reactivity from primary antibodies (not sure what you're referring to in the image).

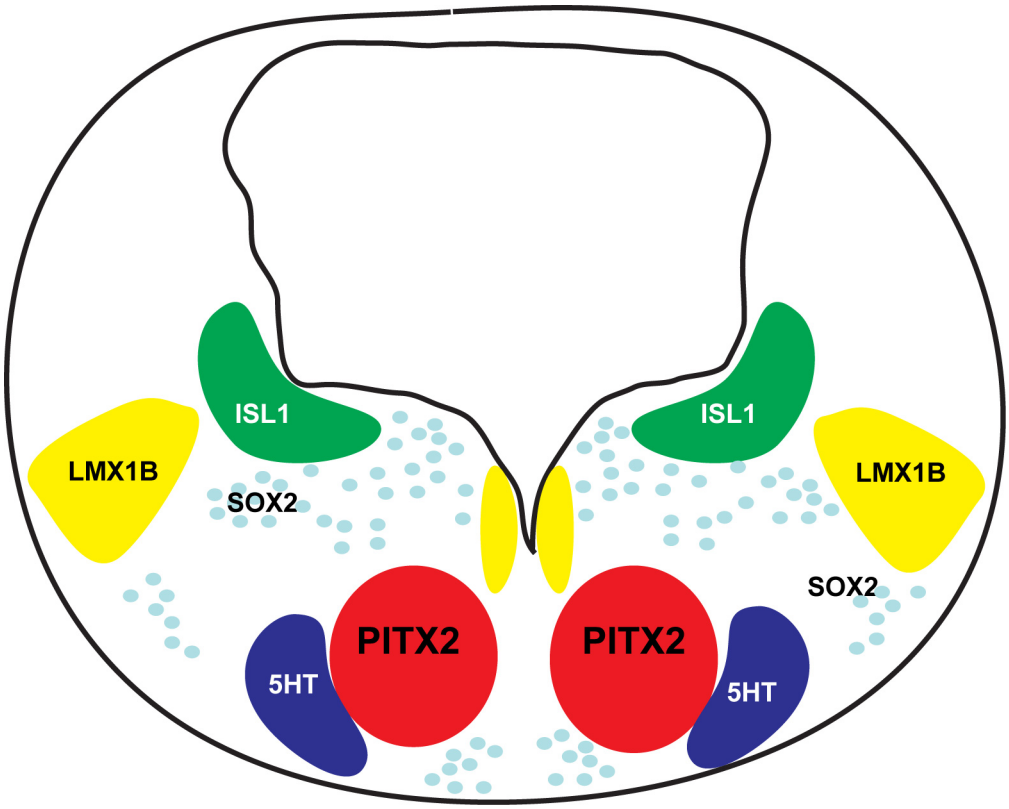


Figure 3.8 Summary of PITX2 expression in rhombomere 1

Schematic of a transverse section of embryonic hindbrain at approximately E14.5. PITX2 expression is shown with respect to transcription factors SOX2 and LMX1B and markers of serotonergic dorsal raphe and cranial motor neurons (ISL1).

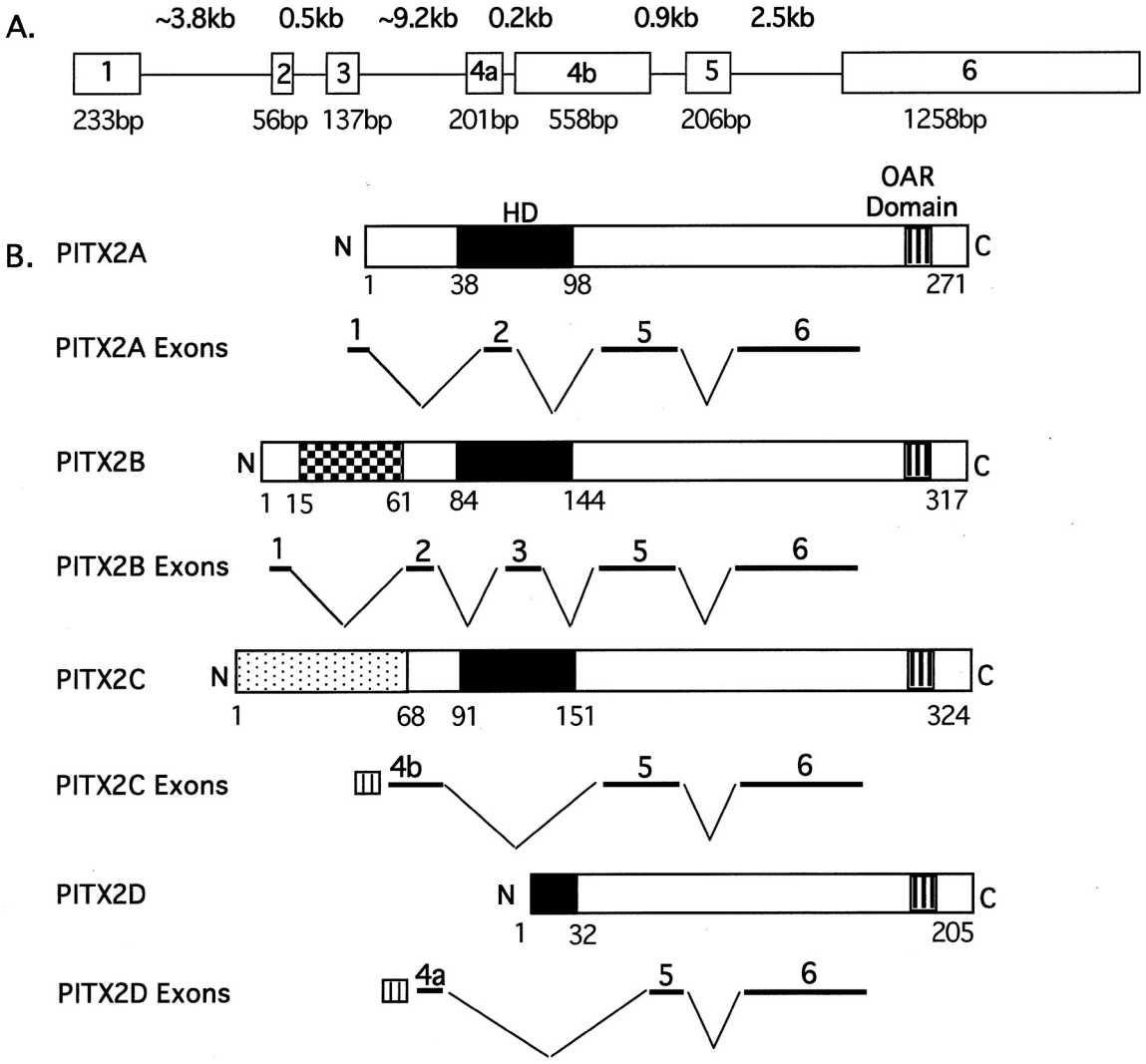


Figure 3.9 Supplementary Figure S1. Major isoforms of *PITX2* in humans.

(A) Genomic organization of the PITX2 gene; top numbers indicate intron sizes, bottom numbers indicate exon sizes. (B) Protein structure of each of the PITX2 isoforms. Solid box – homeodomain; thick striped box – Otp and aristaless (OAR) domain; checkered and stippled boxes – divergent N-terminal region; thin striped box – internal promoter. From Cox et al., 2002.

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Chapter 4

Conclusion

My thesis work has been focused on the role of transcription factors in driving the generation of specific neuronal subtypes in the developing central nervous system. Specifically, I have examined the role of the basic helix-loop-helix transcription factor Bhlhb5 in spinal cord development and have investigated expression of *Pitx2* in the embryonic ventral hindbrain.

Bhlhb5 in spinal cord development

The results presented in this dissertation have contributed to the understanding of generation of distinct neuronal classes within the developing spinal cord. The Olig-related transcription factor Bhlhb5 plays two central roles in patterning of progenitor domains and subsequent differentiation of dI6, V1 and V2 interneurons. Bhlhb5 repressor activity first acts downstream of retinoid signaling and homeodomain proteins to promote the formation of dI6, V1, and V2 interneuron progenitors and their differentiated progeny. In addition, Bhlhb5 is required to organize the spatially-restricted expression of the Notch ligands Jagged1, Dll1, and Dll4 that elicit the formation of specific interneuron populations that form adjacent to Bhlhb5^{+} cells and influence the

global pattern of neuronal differentiation. Through these actions, Bhlhb5 helps transform the spatial information established by morphogen signaling into local cell-cell interactions associated with Notch signaling that control the progression of neurogenesis and extend neuronal diversity within the developing spinal cord

Significance and Future Directions

Studies presented here describe the role of Bhlhb5 in the generation of specific classes of neurons within the developing spinal cord. Bhlhb5 expression also identifies additional diversity in subgroups of postmitotic neurons emerging from these domains. Further characterization of Bhlhb5^{+} and Bhlhb5 negative subgroups of neurons emerging from single progenitor domains is needed in order to determine differences in the complement of transcription factors, neurotransmitters, and axonal projection phenotypes that define discrete neuronal subgroups that are likely to have unique contributions to locomotor circuitry. In this regard, detailed functional studies of specific groups of Bhlhb5-expressing neurons are also needed. Recently, studies have employed conditional ablation of specific molecularly defined interneuron classes to examine the contribution of individual classes of neurons to control of locomotor activity in complex mammalian systems. For example, conditional ablation of *Pitx2* in spinal cord revealed a novel class of excitatory V0 interneurons that project to ipsilateral motor neurons and contribute to control of task-specific regulation of motor neuron activity (Zagoraiou et al., 2009). Similarly, elimination of V2a interneurons revealed a role for normalization of motor neuron bursting and coupling of left-right alternation in motor neuron firing (Crone et al., 2008). Recent studies have made it clear that many more distinct neuronal

populations derive from the cardinal progenitor domains than had previously been appreciated. *Bhlhb5* is expressed in multiple progenitor domains, marks subsets of mature neurons emerging from these domains, and is thus likely to contribute in complex ways to establishment and maintenance of this diversity. The V2a neuronal population, one of the better characterized functional groups in spinal cord, can be further subdivided on the basis of *Bhlhb5* expression, and examination of each subpopulation might further refine understanding of locomotor circuitry. The functional contributions of *Bhlhb5*-expressing interneuron populations have yet to be established but will likely provide interesting insights into the connectivity and function of these complex circuits.

During development of the central nervous system, a limited number of transcription factors is employed in a combinatorial manner to generate a diverse variety of neuronal subtypes. The same transcription factors and accessory proteins are used in different combinations to drive generation of diverse neuronal types. bHLH proteins are particularly complex to study in this regard, in part because they rarely work alone and must cooperate with binding partners to carry out their functions. Loss of function and gain of function analyses are also difficult because manipulation of levels of one protein may alter the balance of cofactor availability for others and these stoichiometric changes may produce a chain of ripple effects. These effects may underlie interpretation of conflicting results that show differential activation or repression in different systems that reflect complexes that may form or be inhibited from forming due to complex interactions with other available factors (Conway et al., 2010). To understand Bhlhb5 function more fully, it will be important to identify its regulatory partners and the components of functional complexes in which it operates in different contexts. Direct

upstream regulatory factors and direct downstream targets for Bhlhb5 have yet to be identified but will be important in understanding the position of Bhlhb5 in the regulatory cascades that operate during the generation of specific cell types. These functional relationships must include an investigation of positive and negative feedback that influence Bhlhb5 expression. Bhlhb5 appears to be able to regulate its own expression (K.S. and B.N. unpublished observations), which is a complicating factor in gain and loss of function studies. Several approaches have been suggested specifically for the study of bHLH proteins to help tease out functionally relevant complexes that may be useful in future studies of Bhlhb5 function. One is the use of tethered bHLH constructs, where two bHLH proteins are translated as a single unit, thus forcing dimerization and preserving stoichiometric ratios and availability of cofactors (Neuhold and Wold, 1993; Castanon et al., 2001; Connerney et al., 2006). In my work, the use of tethered bHLH constructs such as Bhlhb5 with an E-protein or Bhlhb5 with Neurog would provide stricter control over the respective concentrations of these proteins within individual cells and suggest functional complexes that might be relevant *in vivo*. A second approach which has proved valuable in exploring functional relationships among bHLH factors uses replacement constructs in which the coding domains of one bHLH factor are replaced by that of a related factor (Davis et al., 1989; Chien et al., 1996; Parras et al., 2002). For example, we have noted that Bhlhb4 may in part compensate for the absence of Bhlhb5 in mouse spinal cord (K.S. and B.N. unpublished observations) and electroporation experiments in chick have suggested partial functional equivalence. Replacement constructs might provide a means for testing this hypothesis as well as for

determining whether there are different requirements for Bhlhb5 function in different domains.

Consistent with multiple roles attributed to bHLH transcription factors depending on context, Bhlhb5 expression is observed in subsets of neurons with diverse characteristics within the various neuronal tissues in which it is expressed. Further, Bhlhb5 is shown to have different functions within different contexts. Bhlhb5 is expressed in diverse postmitotic neuronal subsets. For example, Bhlhb5 is expressed in a fraction of both excitatory and inhibitory dorsal association neurons in spinal cord that derive from a common lineage (Liu et al., 2007) and in subsets of both amacrine and bipolar cells from divergent lineages in retina (Feng et al., 2006). Bhlhb5 affects cell fate in some brain areas in which it is expressed, such as the caudal sensorimotor cortex, but not in others, such as the rostral and occipital cortex (Joshi et al., 2008). It appears to affect primarily cell fate in dI6 and V2 lineages in spinal cord, but to be more involved in neurogenesis in V0 and V1. In addition, multiple phenotypes result from loss of Bhlhb5 function, including premature neurogenesis in spinal cord, lack of proper cell type specification in spinal cord and cortex, loss of specific postmititotic cell types in retina and cortex, misregulation of axonal projections in the corticospinal motor neurons (CSMN) (Joshi et al., 2008), and increased cell death in sensory neurons of the dorsal horn (Ross et al., 2010). Thus, Bhlhb5 is likely to have multiple functions regulated in diverse ways during development of different neuronal tissues.

It is clear that many details of Bhlhb5 function remain to be elucidated. Intriguingly, in cortex as well as in spinal cord, loss of Bhlhb5 function appears to affect cell types which do not ordinarily express Bhlhb5. In the cortex, loss of Bhlhb5 function

results in failure of caudal CSMNs, which express high levels of Bhlhb5, to enter the medullary pyramidal tract and send projections to spinal cord. Interestingly, although Bhlhb5 is not expressed in rostral CSMNs, axons from these cells also fail to enter the corticospinal tract, suggesting an additional non-cell-autonomous influence of Bhlhb5 on provision of guidance, elongation or maintenance signals (Joshi et al., 2008). Furthermore, Bhlhb5 action in specification of area- and layer-specific cell type identities varies even among cortical areas in which it is highly expressed. For example, the nuclear orphan receptor RORß is expressed in layer IV along the entire rostral-caudal axis and is downregulated in Bhlhb5 nulls in the caudal motor cortex but not the occipital cortex, both areas of high Bhlhb5 expression (Joshi et al., 2008). Similarly, despite Bhlhb5 expression in layers II-V of the somatosensory cortex, the axon guidance molecule Ephrin-A5 is downregulated in layers IV-V but not in layers II-III in the absence of Bhlhb5 (Joshi et al., 2008). These apparently non-cell autonomous roles of Bhlhb5 in spinal cord and cortex remain to be investigated.

Results presented in my studies suggest that Bhlhb5 manipulations have non-cell autonomous effects in spinal cord. Knockdown of Bhlhb5 resulted in a decrease in numbers of cell types with which Bhlhb5 is normally associated as well as a surprising decrease in cell types that do not normally express Bhlhb5. Bhlhb5 was shown to have a general effect on neurogenesis when misexpressed or reduced. Misexpression of Bhlhb5 represses the ability of cells to exit the cell cycle and differentiate, whereas reduction of Bhlhb5 function results in premature cell cycle exit and differentiation across cell types. These general effects on proliferation and differentiation cannot fully explain differential effects on specific neuronal subtypes. Manipulation of Bhlhb5 expression was also found

to affect Notch signaling. This effect was observed through disruptions in the distribution of Notch ligands Dll1 and Jag1 and the Notch modulator Lfng, as well as effects on the Notch target gene Hes5. Further study of the relationship between Bhlhb5 and Notch pathway components might further explain these results. The effects of manipulating Notch ligands on Bhlhb5 and neuronal subtype specification could be examined as can effects on Bhlhb5 expression in Notch ligand mutants.

One of the most important issues that remains to be addressed is the discrepancy between results observed in the our study, which primarily used *in ovo* electroporation techniques in chick to misexpress and knock down Bhlhb5 expression, and the lack of notable ventral spinal cord phenotypes in Bhlhb5-null mice. Overall, the pattern of progenitor domains and subsequent production of ventral interneuron subtypes in Bhlhb5 mice appears comparable to controls; the mutant mice are viable and exhibit no overt motor phenotype (Joshi et al., 2008; Ross et al., 2010). A subset of late-born dorsal interneurons fails to survive in Bhlhb5 mutant mice, resulting in an elevated itch response (Ross et al., 2010), and there may be subtle defects in production of a subset of ventral V2b neurons (B.N., unpublished observations), although this has yet to be thoroughly examined. The lack of an overt phenotype in the ventral spinal cord in Bhlhb5 mutant mice, given the robustness of the effects of Bhlhb5 manipulation on ventral interneuron populations, is puzzling. One possible explanation involves redundancy among bHLH proteins, such that the function of another family member may compensate for the absence of a given protein. In the spinal cord, the highly related protein Bhlhb4 is expressed in a pattern that suggests that it might be redundant with Bhlhb5. This was supported by studies in which misexpression of Bhlhb4 led to effects similar to those

observed with misexpression of Bhlhb5 (K.S. and B.N., unpublished observations). This hypothesis could be further investigated through creation and analysis of *Bhlhb4*;*Bhlhb5* double mutants to determine effects of removal of both bHLH proteins from the developing spinal cord.

Another major difference between Bhlhb5 null mice and the chick electroporation system is timing. Bhlhb5 mice experience chronic loss of Bhlhb5, whereas electroporation results in acute removal of Bhlhb5 function. In the developing spinal cord, multiple signaling systems and transcription factors interact to pattern dorsal-ventral progenitor domains. In the chick embryo, this initial patterning takes place in the presence of endogenous Bhlhb5, prior to misexpression or knockdown. In null mice, however, Bhlhb5 is absent throughout gestation, yet patterning and cell type specification proceed normally. Further study of the effects of Bhlhb5 function would benefit from use of a conditional knockout of Bhlhb5 such that Bhlhb5 function could be removed at specific points during development and effects analyzed. Acute perturbations of this dynamic system of interacting transcription factors may uncover effects that are not evident in the germline null animal. It is of course possible that differences between species (chick vs. mouse) account for the observed differences. However, this seems less likely than other explanations due to the highly conserved sequence and expression patterns between the two proteins.

Pitx2 in development of spinal cord and hindbrain

Data reported in this thesis represent an initial attempt to characterize Pitx2-expressing cells in spinal cord and hindbrain. A thorough description of the

developmental origin, characteristics and function of small population of Pitx2^{+} cells in the spinal cord has recently been published (Zagoraiou et al., 2009; Enjin et al., 2010). These published data are consistent with our preliminary work identifying Pitx2^{+} cells in spinal cord as a subset of V0 interneurons. In addition, recently published work demonstrates that some Pitx2^{+} spinal cord cells are cholinergic interneurons that project to motor neurons and are the sole source of cholinergic synapses that form the distinctive C boutons. In addition to these cholinergic Pitx2-expressing neurons, some Pitx2^{+} neurons are glutamatergic (Zagoraiou et al., 2009). A major implication of these studies is that greater diversity in neuronal subtypes is created from the cardinal progenitor domains within the developing spinal cord than had previously been appreciated. Possibly hundreds of molecularly and functional diverse types of neurons exist, far more than can be identified by currently known molecular, physiological, or anatomical markers (Zagoraiou et al., 2009).

In the developing hindbrain, Pitx2 expression begins at about E9.5 in mouse and persists throughout embryonic development. A cluster of Pitx2^{+} cells occupies a ventral position in r1, the most anterior of subdivisions in the developing hindbrain. The precise identity of this neuron cluster has not yet been determined; however, these cells do not appear to belong to the most characterized subpopulations or nuclei in r1. They may represent a heretofore uncharacterized nucleus or a coherent set of local neurons that modulate signals from one or more of the many neuronal tracts that pass through the hindbrain. Although further investigation is needed, at least some of these cells at E12.5 are GABAergic (Martin et al., 2002) and may provide inhibitory modulation for local hindbrain circuits.

Significance and Future Directions

Several open questions remain regarding the nature and function of Pitx2^{+} cells in spinal cord. Two subpopulations of cells that express Pitx2 were found to exist in spinal cord, the cholinergic V0C and the glutamatergic V0G subtypes. Currently published research has characterized in detail only the V0C subtype (Zagoraiou et al., 2009). The functional contribution of V0G neurons to locomotor circuits within the spinal cord has yet to be investigated. The mechanisms by which neurons with vastly different characteristics and functions are created from a common progenitor pool also have yet to be examined. V0 neurons exhibit at least four different neurotransmitter phenotypes, (both excitatory and inhibitory) widely varying projection trajectories (ipsilateral and contralateral), and different targets, including motor neurons and interneurons. Whether this diversity of neuronal types is generated in a hierarchical, sequential, or parallel manner has yet to be determined. In addition, the pathways that direct such diversity need to be identified. In the case of V2 interneuron subtypes, the decision between the V2a and V2b fate is mediated by the Notch signaling pathway, a common mechanism driving binary cell fate choice (Del Barrio et al., 2007; Peng et al., 2007). Notch signaling also plays a temporal role in the switch between neuronal and glial fates in Olig2^{+} precursors (Louvi and Artavanis-Tsakonas, 2006). Notch signaling could underlie the segregation of p0 progeny into discrete classes. In other cases, such as diversity between V2 and motor neurons and within motor neuron subtypes is coordinated by regulatory networks between various transcriptional activators and repressors that act to segregate cell fates using related but distinct complexes to drive transcription (Lee et al.,

2008; Rousso et al., 2008). An analysis of transcriptional cascades, upstream regulatory regions, and biochemical composition of regulatory transcriptional elements might provide valuable insights into how these diverse subtypes are generated in characteristic numbers and patterns.

No loss of function analysis has yet been presented to determine the fate of $Pitx2^{+}$ V0 interneurons in the absence of Pitx2. Selective elimination of ChAT from V0C neurons did not disrupt the formation or organization of C bouton synapses on motor neurons (Zagoraiou et al., 2009). The same approach could be used to study the V0G subpopulation. This type of analysis might yield vastly different results, since in contrast to acetylcholine, glutamate has been shown to influence axonal guidance and synapse formation (Ruediger and Bolz, 2007). The effects of complete elimination of Pitx2 from V0 neurons could be investigated through examination of spinal neuron formation in embryos obtained from the crossing of a mouse line containing a conditional Pitx2 deletion allele (Gage et al., 1999; Skidmore et al., 2008) with a mouse expressing Cre recombinase under control of the p0 marker Dbx1 (Pierani et al., 2001). Potential fate switches could be investigated by including a $Pitx2^{LacZ}$ allele in the V0-Pitx2 conditional null in order to permanently mark presumptive $Pitx2^{+}$ cells and determine their fate in the absence of Pitx2. Using these approaches, the functional significance of Pitx2 expression in V0 spinal neurons could be further examined.

Characterization of the identity of Pitx2-expressing cells in r1 of the developing hindbrain is incomplete. Many questions remain regarding the identity and function of the group of $Pitx2^{+}$ cells in ventral r1. Foremost is the determination of the relationship of this discrete and organized cluster of cells to known nuclei and structures in

developing r1. Examination of the transcriptional, neurotransmitter and anatomical characteristics of these cells in comparison to known nuclei and tracts will determine whether this group of cells fits within currently characterized domains or whether it defines a unique structure or functional group within the rostralmost region of the hindbrain. A developmental progression from earliest expression at E9.5 through the early postnatal period when the hindbrain is still developing should be completed to determine changes in *Pitx2* expression with respect to cell movements and organization within this area. Existing mouse models can be used to further determine the identity and function of *Pitx2*-expressing cells in ventral r1. Cells expressing *Pitx2* can be permanently marked by creating compound heterozygous animals that express $Pitx2^{cre/+}$ or $Pitx2^{cre/-}$ and a nuclear- or cytoplasmic-localized ß-galactosidase. This approach has been used successfully to examine the function of *Pitx2* and changes that occur in the absence of *Pitx2* function in other brain regions (Skidmore et al., 2008; Waite et al., 2010). We have begun analysis of compound heterozygous animals, but analysis of *Pitx2* null mutants remains to be done. Results from these studies would contribute information about a previously uncharacterized neuronal population as well as increase understanding of neuronal organization within r1 in the developing hindbrain.

Results presented herein underscore the need for continued refinement of knowledge regarding organization of specific neuronal subgroups particularly in ventral r1 of the developing hindbrain. While much attention has been directed to understanding and characterizing the developmental processes that lead to formation of the cerebellum from dorsal r1, ventral r1 remains a much understudied area. Characterized generally as being composed of a loose collection of indistinct nuclei, more details about the

characteristics of molecularly and anatomically defined groups of cells are much needed. An elucidation of the developmental origins of distinct neuronal groups through lineage tracing analyses is particularly important as contradictory information exists as to the origin of many of the cells that form the ventral rostral hindbrain nuclei. Also lacking are studies that directly link the developmentally transient rhombomere structures to their counterparts in mature brain. The cerebellum is a brain structure with complex organization and progress has been made in defining the developmental origins, identities, and movements of neurons that give rise to its mature form. The processes involved in generating derivatives of the more caudal rhombomeres, such as the origins, development, and organization of specific cranial nerves, have also received attention. However, with the exception of a very few limited cell groups, the derivation, characteristics, and organization of neurons within ventral r1 remain largely unknown.

Summary

The studies reported in this dissertation highlight an increased understanding of the diversity of neuronal subtypes that make up the vertebrate central nervous system. It is increasingly clear that combinatorial interactions of multiple transcription factors generate specific neuronal subtypes and that these interactions may be influenced by the cellular context in which they take place. The answer to the fundamental question of how generation of neuronal diversity in developmental neurobiology occurs will depend on elucidation of the many interactions among these multiple factors. This will involve decoding the combinatorial activities of partially overlapping transcriptional activators and regulators in establishing sharp regionalization and coordination of post-mitotic cell

fates required for construction of the functioning CNS during development. Progenitors expressing regulatory transcription factors establish general domain identities and additional transcription factors and signaling systems are involved in establishment of increasingly specific identities in postmitotic domain-restricted neurons. By acting combinatorially, genes that are expressed in diverse domains may have diverse functions that depend on context. From early studies of Drosophila segmentation and neuronal development to increasingly complex illustrations of interactions within the developing vertebrate nervous system, some of these interactions are being revealed. In addition, many of the processes described herein are also observed in other neural and non-neural tissues, indicating that these results provide insights relevant to development in diverse organ systems.

In addition to contributions to the understanding of normal developmental processes, elucidation of the roles of proteins such as Bhlhb5 and Pitx2 that contribute to specific neuronal classes can provide valuable direction for therapeutic treatment of devastating neurological diseases. Much of the enthusiasm for stem cell research has come from the promise of pluripotent stem cells to generate any or all of the many types of neurons that may be damaged or missing in disease. Appreciation of the diversity of neuronal types that exist and elucidation of the process of how these hundreds of specific types of neurons are produced in proper positional, spatial, and temporal location is crucial to developing strategies to treat or repair neurons that are damaged or dysfunctional due to degenerative or traumatic injury. Clearly, the acquisition of particular neuronal identities and their establishment into functional neuronal circuits requires more than knowledge of the complement of transcription factors required to

induce a particular cell type. Capitalization on processes that recapitulate normal neural development in context can be a vital strategy for investigating uses of neural stem cells for CNS regeneration and repair (Madhavan and Collier, 2010; Okano, 2010).

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